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Departamento de Biología

"Caracterización de la ruta de N-glicosilación de proteínas en el sistema de endomembranas del alga verde *Chlamydomonas reinhardtii.* Distribución al cloroplasto de N-glicoproteínas."

"Characterization of the N-glycosylation pathway through the the endomembrane system in the green alga *Chlamydomonas reinhardtii*. N-glycoprotein trafficking to the chloroplast"

Tesis doctoral

Doctoral Thesis

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A Arse
A mi familia

Índice/index

ABREVIATURAS / *ABBREVIATIONS*

Abreviaturas / Abreviattions

α-ManI: α-Manosidasa I/ α-Mannosidase I.

α-ManII: α-Manosidasa II/ α-Mannosidase II.

α(1,3)FT: α(1,3)-fucosiltranserasa/ α(1,3)-fucosyltransferase

α(1,4)FT: α(1,4- fucosiltranserasa/ α(1,4)-fucosyltransferase

α(1,6)FT: α(1,6)-fucosiltransferasa/ α(1,6)-fucosyltransferase

β(1,2)XT: β(1,2)-xilosiltransferasa/ β(1,2)-xylosyltransferase

β(1,3)GalT: β(1,3)-galactosiltransferasa/ β(1,3)-galactosyltransferase

2AB: 2-Aminobenzamida/2-Aminobenzamide

aa : Aminoácidos/ Amino acids

ADN /DNA: Ácido dexosirribonucleico/ Deoxyribonucleic acid

ADNc/DNAc: Ácido dexosirribonucleico copia/ Complementary DNA

AAL: Lectina biotinilada Aleuria Aurantia /Biotinylated Aleuria Aurantia Lectin

ALG: Asparagine-linked-glycosylation

amiRNA: microRNA artificial / Artificial microRNA

ARN/RNA: Ácido ribonucleico/ RiboNucleic Acid

Asn: Asparragina/Asparagine

BFA: Brefeldina A/Brefeldin A

BSA: Albúmina bovina/ Bovine serum albumin

CAZy: Carbohydrate Active enzyme

Con A: Concanavalina A/ Concanavalin A

CTAB: Bromuro de cetiltrimetilamonio /Cetyl trimethylammonium bromide

DEPC: Dietilpirocarbonato/ Diethyl pyrocarbonate

DMSO: Dimetil sulfóxido/ Dimethyl Sulfoxide

dNTPs: Desoxinucleótidos trifosfato / Deoxynucleotides triphosphate

DTT: Ditiotritol/ Ditiotreitol

Endo H: Endoglicosidasa H/ *Endoglycosidase H*

Gal: Galactosa/Galactose

Glc: Glucosa/glucose

GlcNAc: N-acetilglucosamina/ N-acetylglucosamine

GNTI: N-acetilglucosaminiltransferasa I/N-acetylglucosaminyltransferase I

GNTII: N-acetilglucosaminiltransferasa II/ N-acetylglucosaminyltransferase II

HA: Hemoaglutinina/ *hemagglutinin*

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPR: Hidroxipiruvato reductasa/ horseradish peroxidase

HS2: Medio rico en sales/High Salts medium

Kb: Kilo base

kDa :Kilo Dalton

Man: Manosa/ Mannose

MALDI-TOF MS: Desorción / ionización láser asistida por matriz-espectrometría de masas de tiempo de vuelo / Matrix-Assisted Laser Desorption Ionisation-Time Of Flight mass spectra

Met: Metionina/ Methionine

MS: Espectrometría de masas / Mass spectrometry

NCBI: Centro Nacional para la Información Biotecnológica / National Center for Biotechnology Information

ORF: Marco de lectura abierto/ Open reading frame

OST: Oligosaccharyltransferase

pb: Pares de bases/ Base pairs

PCR: Reacción en cadena de la polimerasa/ polymerase chain reaction

PEB: Tampón de extracción de proteínas/ Protein Extraction Buffer

PEG: Polietilenglicol/ *Polyethylene glycol*

PIC: Coctel de inhibidores de proteasas/ Protease *Inhibitor Cocktail*

PNGasa F: N-peptidilendoglicosidasa F / Peptide-N-glycosidase F

PNGasa A: N-peptidilendoglicosidasa A/ Peptide-N-glycosidase A

POX: Peroxidasa/Peroxidase

RACE: Amplificación rápida de extremos ADNc /Rapid amplification od cDNA Ends

RE/ER: Reticulo endoplásmico/ Endoplasmic reticulum

Rpm: Revoluciones por minuto/ Revolution per minutes

RuBisCO: ribulosa-1,5-bisfosfato carboxilasa oxigenasa / ribulose-1,5-biphosphate carboxylase oxygenase

SAP: Fosfatasa alcalina procedente de gamba/ Shrimp alkaline Phosphatase

SDS: Dodecilsulfato sódico/ Sodium dodecyl sulphate

SP: Péptido señal/Signal peptide

TAE: Tampón Tris-Acético-EDTA/ *tris-acetic EDTA buffer*

TAP: Tris-Acetato-Fosfato/Tris-acetic-phosphate

Tm: Temperatura de anillamiento/ *melting temperature*

TP: Péptido tránsito/ Transit peptide

TRIS: Tris(hidroximetil)aminometano /Tris(hydroxymethyl)aminomethane

UTR: Región transcrita pero no traducida/ untranslated region

* Abbreviations other than those listed here are defined in their first mention in the text.

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I. INTRODUCCIÓN

I. Introducción.

1. Transporte de proteínas al cloroplasto en plantas. Una nueva ruta de transporte de N-glicoproteínas a través del sistema de endomembranas.

Los cloroplastos son orgánulos semi-autónomos presentes en las células vegetales. Estos orgánulos presentan compartimentos diferentes y realizan procesos esenciales como el proceso fotosintético, además de síntesis de ácidos grasos, de algunos aminoácidos y asimilación de nutrientes.

Los cloroplastos se originaron por un proceso endosimbiótico en el cual una célula procariota fotosintética fue fagocitada por un huésped eucariota. Durante el curso de la evolución, la mayoría de los genes del endosimbionte fueron transferidos al núcleo del huésped (Delwiche, 1999). Por lo que, aunque los cloroplastos poseen su propio ADN y ribosomas, la mayoría de las proteínas cloroplásticas se codifican en el genoma nuclear y tienen que ser importadas al cloroplasto (Keegstra y Cline, 1999; Leister, 2003; Jarvis, 2008).

Hasta la publicación en el año 2005 del trabajo de Villarejo y colaboradores, estaba establecido que las proteínas cloroplásticas codificadas en el núcleo eran sintetizadas en el citosol como precursores con una señal de distribución en el extremo amino terminal llamada péptido tránsito (*transit peptide,* TP) que dirige la proteína al cloroplasto en un proceso post-traduccional (Gutensohn y col., 2005; Jarvis, 2008). En general, el proceso de transporte de los precursores a través de las membranas del cloroplasto es mediado por dos complejos oligoméricos llamados Complejo Toc (translocón en la membrana externa, *outer* en inglés, cloroplástica) y Complejo Tic (translocón en la membrana interna cloroplástica) (Jarvis y Robinson, 2004; Gutenshon y col., 2005) (Figura I).

La mayoría de las proteínas codificadas en el núcleo y con destino final al cloroplasto siguen esta ruta. Sin embargo, estudios recientes del proteoma del cloroplasto habían revelado la existencia de proteínas que carecían del TP (Kleffam y col., 2004) y proteínas con un péptido señal (*signal peptide,* SP) para el retículo endoplasmático (RE) (Kilian y Kroth, 2005), lo que sugería que la distribución y transporte de proteínas al cloroplasto podría ser más compleja de lo que se esperaba y que el complejo Toc/Tic no sería el único sistema de transporte.

En el trabajo realizado por Villarejo y col. (2005) se demostró la existencia en *Arabidopsis thaliana* de una ruta alternativa de transporte y distribución de proteínas con destino al cloroplasto, a través del sistema de endomembranas (RE y Golgi). Los resultados obtenidos por Villarejo y col. (2005) muestran que una carbónico anhidrasa denominada CAH 1, se localiza específicamente en el estroma del cloroplasto de *Arabidopsis* a pesar de la presencia en su secuencia de un SP típico para el RE. Estos autores demostraron que CAH 1 es una proteína que se transporta primero al RE donde se elimina el SP y donde la proteína adquiere N-glicanos. A partir del RE, la proteína se

distribuye al Golgi donde se va a ver sometida a la acción de distintas enzimas que van a modificar su N-glicano transformándolo en el N-glicano complejo típico de plantas, el cual contiene residuos de α(1,3)-fucosa y β(1,2)-xilosa característicos. Finalmente, la proteína se transporta desde el Golgi a su destino final, el cloroplasto, por un mecanismo aún desconocido (Figura I).

Estos datos indicaban que al menos en *A.thaliana* el proteoma plastídico contiene, además de proteínas transportadas a través del complejo Toc/Tic y proteínas sintetizadas en el propio cloroplasto, glicoproteínas distribuidas a través del sistema de endomembranas (RE y Golgi).

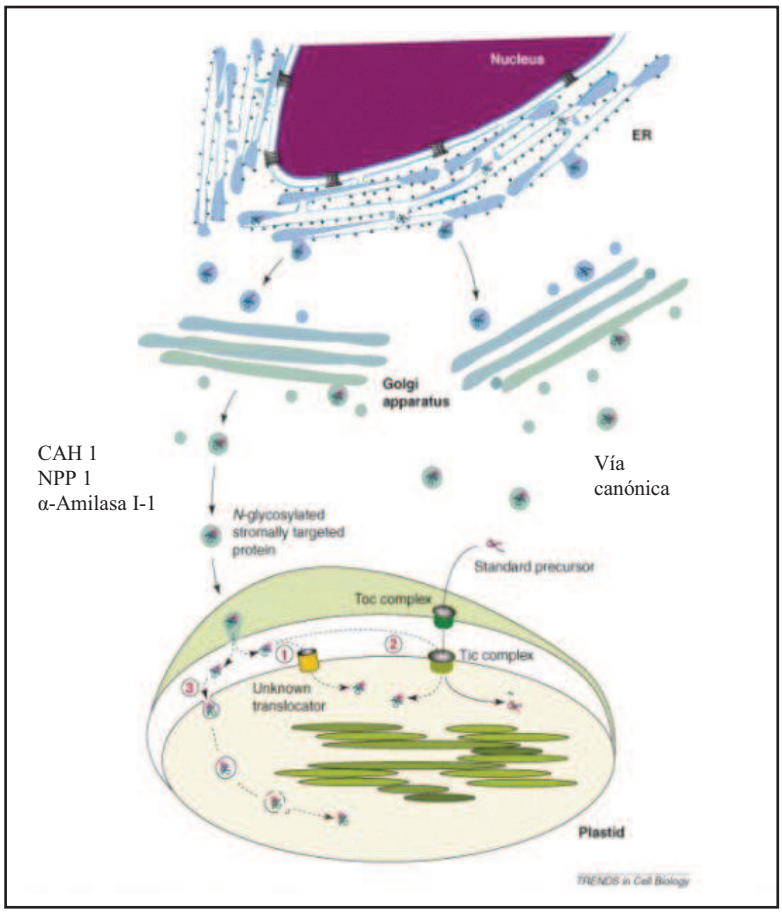


Figura I. Esquema de la nueva ruta de proteínas glicosiladas hacia el cloroplasto.
Figura adaptada del artículo de Radhamony y Theg (2006).

Este es el primer ejemplo conocido en plantas de la existencia de una ruta de transporte de proteínas al cloroplasto a través del sistema de endomembranas y de la presencia de glicoproteínas en orgánulos de origen endosimbionte como el cloroplasto. Tiene gran relevancia tanto desde el punto de vista del estudio de un proceso biológico básico como desde el punto de vista biotecnológico.

Recientemente, otros autores han descrito la presencia de otras proteínas glicosiladas en los cloroplastos de arroz y se han caracterizado la nucleótido pirofosfatasa/fosfodiesterasa (NPP1) (Nanjo y col., 2006) y la α-Amilasa I -1 en este

organismo (Asatsuma y col., 2005). Ambos grupos han demostrado que dichas glicoproteínas siguen una ruta a través del sistema de endomembranas similar a la descrita por Villarejo y col. (2005) para la glicoproteína CAH 1 de *Arabidopsis* (Figura I).

Se confirma de esta manera que el proteoma del cloroplasto contiene proteínas N-glicosiladas y que existe una ruta alternativa de transporte y distribución para proteínas con destino al cloroplasto, a través de lo que se conoce como el sistema de endomembranas (Radhamony and Theg, 2006) tanto en plantas monocotiledóneas como en dicotiledóneas, sin embargo no se había comprobado su existencia en organismos fotosintéticos más primitivos.

2. El proceso de N-glicosilación de proteínas en plantas.

La N-glicosilación es una de las modificaciones post-traduccionales más importantes en el proceso de maduración de las proteínas en eucariotas. Este proceso está parcialmente conservado en todos los organismos eucarióticos y se produce de forma secuencial y ordenada, primero en el retículo endoplásmico y después con la maduración en el aparato de Golgi (Figura II).

2.1 N-glicosilación de proteínas en el Retículo endoplásmico.

Como en otros eucariotas, la N-glicosilación de proteínas de plantas comienza cuando la proteína penetra en el primer compartimento del sistema de endomembranas, el RE, mediante un proceso simultáneo a la traducción. Una vez dentro sufre la primera modificación, que consiste en la trasferencia de un oligosacárido precursor cuya estructura está constituida por tres glucosas, nueve manosas y dos N-acetilglucosaminas ($Glc_3Man_9GlcNAc_2$), en el extremo amino terminal de un residuo de Asparragina (Asn) (Vitale y col., 1993) (Figura II). El precursor posteriormente va a ser modificado mediante la acción de glicosidasas y glicosiltransferasas localizadas en el RE y en el aparato Golgi durante su transporte (Hammond y col., 1994).

En el retículo endoplásmico, muy tempranamente, las unidades de glucosa terminales del precursor Glc3Man9GlcNAc2 son eliminadas mediante la acción de las glicosidasas I y II (Szumilo y col., 1986a; Kaushal y col., 1990a; Lerouge y col., 1998; Bardor y col., 2009). Este proceso produce un N-glicano rico en manosa (Man9GlcNAc2) característico del RE (Figura II).

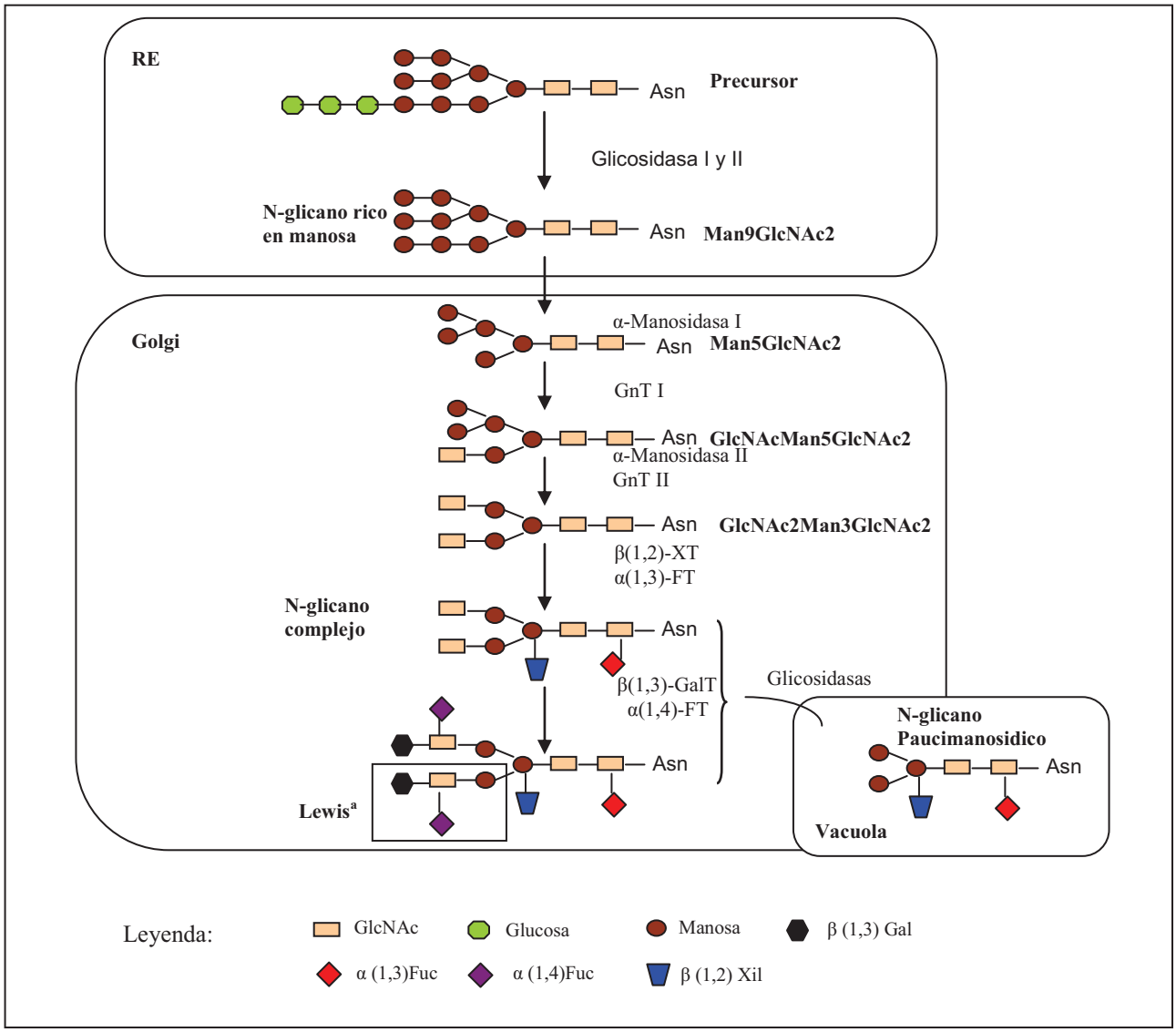


Figura II. Proceso de N-glicosilación de proteínas en plantas. La N-glicosilación es una de las modificaciones post-traduccionales más importantes en el proceso de maduración de las proteínas en eucariotas. Comienza en el RE y continúa con la maduración en el Golgi. En el proceso están implicadas numerosas enzimas dando lugar a diferentes estructuras de N-glicanos como son los: N-glicanos ricos en manosa, N-glicanos complejos, estructura de Lewis, entre otros. *Imagen adaptada del articulo de Lerouge y col., 1998.*

2.2 Procesamiento y modificación de N-glicanos en el aparato de Golgi.

A medida que las glicoproteínas se mueven del RE al Golgi, el N-glicano adquirido en el RE va a ser modificado dando lugar a los N-glicanos complejos de plantas. La formación de los N-glicanos complejos asociados a las glicoproteínas que se transportan a través del Golgi tiene lugar gracias a la acción de un conjunto de enzimas que actúan en serie modificando el N-glicano rico en manosa formado en el RE (Lerouge y col., 1998; Bardor y col., 2009) (Figura II).

En plantas, la primera modificación del N-glicano rico en manosa consiste en la eliminación de 1 a 4 residuos de manosa mediante la acción de la α-manosidasa I (α-Man I) pasando el precursor $Man_9GlcNAc_2$ a $Man_5GlcNAc_2$ (Strum y col., 1987, Szumilo y col., 1986b). A continuación, la adición de un primer residuo de N-acetilglucosamina formando un enlace α(1,3) sobre una manosa, se produce por la acción de la enzima N-acetilglucosaminiltransferasa I (GnT I), dando lugar a la estructura $GlcNAcMan_5GlcNaAc_2$ (Figura II). Este paso catalizado por GnT I se considera el inicio de la ruta de biosíntesis del N-glicano complejo de las glicoproteínas de plantas y el fallo en la actividad de esta enzima bloquea la acción del resto de enzimas de la ruta (Johnson and Chrispeels, 1987; Tekuza y col., 1992; Strasser y col., 1999a; Bakker y col., 1999).

De hecho, el mutante *cgl1* de *Arabidopsis thaliana* carece de GnT I y es incapaz de sintetizar glicanos complejos. Como consecuencia, se produce la acumulación de estructuras oligomanosídicas $Man_5GlcNAc_2$ (von Schaewen y col., 1993). Este mutante confirma el requerimiento de dicha enzima para la formación de los N-glicanos complejos. Sin embargo, no presenta un fenotipo diferente al del silvestre, son viables y fértiles completando su ciclo vital bajo condiciones de laboratorio. Solo recientemente se ha demostrado que en condiciones extremas de salinidad se produce letalidad (Kang y col., 2008).

Tras la acción de GnT I, dos residuos de manosa son eliminados por la acción de la α-manosidasa II (α-Man II) (Strasser y col., 2006) como paso previo a la adición de otro residuo de N-acetilglucosamina transferido por la N-acetilglucosamiltransferasa II (GnT II) formando un enlace α(1,6) sobre otra manosa distal (Johnson y Chrispeels, 1987; Kausha y col., 1990b; Tezuka y col., 1992; Strasser y col., 1999b; Chen y col., 2005) (Figura II).

En este punto, se añade al N-glicano con estructura $GlcNAc_2Man_3GlcNAc_2$ un residuo de fucosa mediante enlace α(1,3) en la N-acetilglucosamina proximal del núcleo de quitobiosa (constituido por las dos N-acetilglucosaminas que constituyen la unión a la Asn proteíca) del N-glicano debido a la acción de α(1,3)-fucosiltransferasa (Leiter y col., 1999; Wilson y col., 2001a; Sourrouille y col., 2008) y una xilosa en enlace β(1,2) debido a la acción de β(1,2)-xilosiltransferasa (Strasser y col., 2004; Sourrouille y col., 2008) (Figura II). Los estudios sobre la especificidad de sustrato de α(1,3)-fucosiltransferasas y β(1,2)-xilosiltransferasas demuestran que la presencia de un residuo terminal de N-acetilglucosamina es un pre-requisito clave para la transferencia de α(1,3)-fucosa y β(1,2)-xilosa (Johson y col., 1987, Tezuka y col., 1992, Staudacher y col., 1995; Zeng y col., 1997; Lerouge y col., 1998; Bardor y col., 2009). La especificidad de aceptor para α(1,3)-fucosiltransferasas y β(1,2)-xilosiltransferasas no se ve afectada por la ausencia de residuos de xilosa o fucosa respectivamente y cabe destacar que las glicoproteínas de plantas pueden llevar residuos de α(1,3)-fucosa y β(1,2)-xilosa de manera independiente (Rayon y col., 1998). Se ha demostrado que la

β(1,2) xilosilación ocurre en las cisternas medias del Golgi mientras que la α(1,3) fucosilación ocurre en las cisternas trans (Fitchette-Lainé y col., 1997).

En *Arabidopsis thaliana*, cada una de las enzimas implicadas en el proceso de N-glicosilación en el Golgi están codificadas por un único gen, exceptuando la α(1,3)-fucosiltransferasa que está codificada por dos genes diferentes (*ft11 y ft12*) (Strasser y col., 2004). Algunos autores, como Wilson y col. (2001) postularon que el gen *ft12* es un producto redundante de la duplicación del gen original *ft11* y que su actividad no era necesaria para llevar a cabo la fucosilación en α(1,3). Sin embargo, estudios posteriores (Bakker y col., 2001a; Strasser y col., 2004, Forth y col., datos no publicados) indican claramente que ambos genes son funcionalmente activos y sus productos necesarios para una óptima fucosilación.

Recientemente se han obtenido mutantes de *Arabidopsis thaliana* deficientes en actividades β(1,2)-xilosiltransferasa, α(1,3)-fucosiltransferasa o en ambas enzimas a la vez, (Strasser y col., 2004) obteniendo glicanos con dos residuos de N-acetilglucosamina terminales y sin residuos de fucosa ni de xilosa (GlcNAc2Man3GlcNAc2). Este mutante es viable y no parece presentar un fenotipo diferente al del silvestre.

Tras la adición de fucosa y xilosa, el N-glicano complejo puede ser modificado por adición de galactosas y fucosas terminales, conformando la secuencia Galβ(1-3)(Fucα1-4)GlnNAc, conocida como antígeno de Lewis^{a} (Le^{a}) gracias a la actividad de la β (1,3)-galactosiltransferasa (β(1,3)-GalT) y la α(1,4)-fucosiltransferasa (α(1,4)-FT) en el residuo terminal de N-acetilglucosamina del glicano complejo (Fitchette-Lainé y col., 1997; Melo y col., 1997; Fitchette y col., 1999; Wilson y col., 2001; Bakker y col., 2001a; Strasser y col., 2007) (Figura 2). El estudio de especificidad de sustrato de la α(1,4)-FT ha mostrado que esta enzima transfiere específicamente fucosa desde GDP-Fucosa a Galβ(1-3)-GlcNAc (Crawley y col., 1989; Fitchette-Lainé y col., 1997; Melo y col., 1997).

Se han clonado diferentes genes que codifican glicosiltransferasas implicados en el procesamiento y maduración de los N-glicanos de plantas:

- GnT I de tabaco (Strasser y col., 1999a); *Arabidopsis thaliana* (Bakker y col., 1999) y patata (Wenderoth y von Schaewen, 2000).
- GnT II de *Arabidopsis thaliana* (Strasser y col, 1999b).
- α(1,3)-FT de judía (Leiter y col., 1999); *Arabidospis thaliana* (Wilson y col., 2001b) y alfalfa (Sourrouille y col., 2008).
- β(1,2)-XT de *Arabidopsis thaliana* (Starsser y col., 2004) y alfalfa (Sourrouille y col., 2008).
- α(1,4)-FT de *Arabidopsis thaliana* (Bakker y col., 2001a, Wilson, 2001c).
- β(1,3)-GalT de *Arabidopsis thaliana* (Strasser y col., 2007).

Todas estas enzimas son proteínas de membrana tipo II como las observadas para las glicosiltransferasas de Golgi de levaduras y mamíferos que se caracterizan por presentar una estructura del constituida por un corto NH_2-terminal citoplasmático, un dominio transmembrana, una región de tallo y un gran dominio catalítico COOH-terminal que comprende motivos peptídicos conservados. (Martin y col., 1997; Breton y col., 1998; Grabenhorst y Conradt, 1999; Holmes y col., 2000; Chazalet y col., 2001).

2.3 Modificaciones de los N-glicanos más allá del Golgi.

Después de la maduración en el retículo, Golgi y en compartimentos post-Golgi las glicoproteínas pueden ser modificadas durante su transporte hacia el compartimento final o en el mismo compartimento final (Lerouge y col., 1998; Chen y col., 2005; Bardor y col., 2009), adquiriendo distintas estructuras dependiendo de que el destino sea extracelular, membrana plasmática o vacuola. Muchas glicoproteínas vacuolares se caracterizan por tener un N-glicano conteniendo residuos de fucosa y/o xilosa pero desprovisto de residuos de N-acetilglucosamina terminales (Figura II). Este N-glicano es denominado N-glicano paucimanosídico. Teniendo en cuenta que la presencia de residuos de N-acetilglucosamina terminal en N-glicanos es un pre-requisito para la transferencia de residuos de α(1,3)-fucosa y β(1,2)-xilosa, los N-glicanos paucimanosídicos pueden producirse únicamente durante el proceso de degradación post-Golgi de los N-glicanos complejos. También podrían producirse a partir de glicanos de Lewis[a] por acción de exoglicosidasas como han postulado Lerouge y col. (1998) (Figura II).

El estudio de la estructura de las glicoproteínas demuestra que existe gran heterogeneidad en el proceso de N-glicosilación en plantas. Esta heterogeneidad se observa a tres niveles diferentes: el número de glicanos presentes en la proteína, la cantidad de modificaciones en la misma glicoproteína y la heterogeneidad de las estructuras en el mismo lugar de N-glicosilación (Lerouge y col., 1998; Bardor y col., 2009). Esta heterogeneidad resultaría de las modificaciones parciales llevadas a cabo por enzimas del Golgi y de la degradación de glicanos por exoglicosidasas en los compartimentos donde las glicoproteínas se acumulan.

3. El proceso de N-glicosilación de proteínas en otros organismos.

Como anteriormente hemos mencionado, la síntesis de los N-glicanos ricos en manosa en el RE es un proceso conservado y común para plantas, animales e insectos. Sin embargo, la maduración de los N-glicanos en el Golgi ha dado lugar a una variedad de estructuras complejas específicas en cada organismo (Wilson, 2002). Es en este punto donde plantas e invertebrados divergen de vertebrados en la biosíntesis de los N-glicanos complejos (Fabini y Wilson, 2001).

En mamíferos, se produce una primera diferencia en el RE con la actuación de una manosidasa del RE, que elimina un residuo de manosa, dando lugar a $Man_8GlcNAc_2$. Esta manosidasa del RE no se ha descrito en plantas (Lerouge y col., 1998). En el Golgi, la maduración de los N-glicanos es diferente con respecto a plantas. Los N-glicanos complejos de mamíferos no presentan residuos de xilosa, aunque sí presentan residuos de fucosa unidos por enlace α(1,6) a la N-acetilglucosamina proximal del "*core*" de quitobiosa. Los N-glicanos complejos de mamíferos presentan también residuos de galactosas en enlace β(1,4) sobre las N-acetilglucosaminas terminales gracias a la acción de la enzima β(1,4)-galactosiltransferasa. Y unidas a estas galactosas terminales presentan residuos de ácido siálico (Figura III).

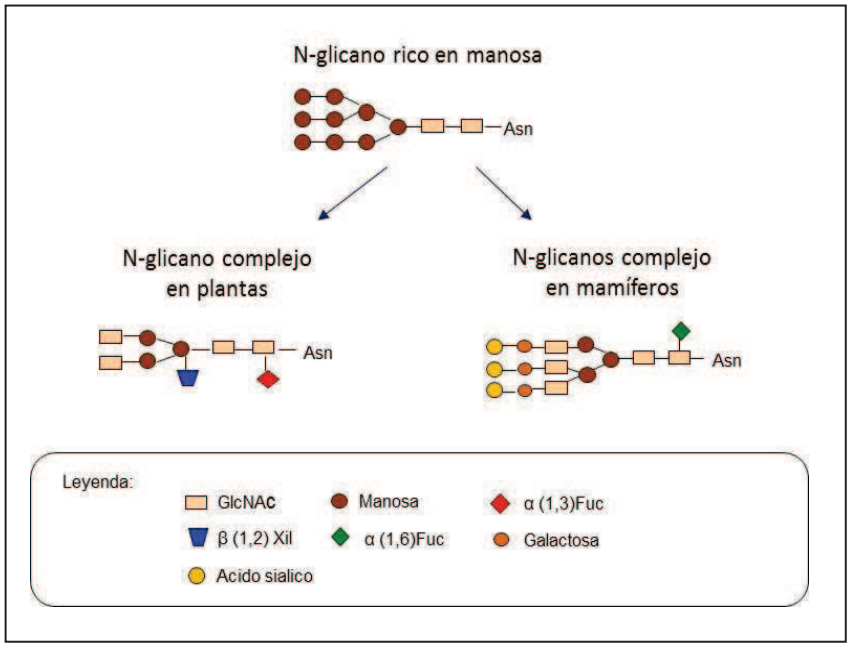


Figura III. Comparación de N-glicanos complejos de plantas y mamiferos. Los N-glicanos complejos de plantas presentan residuos de fucosa en enlace α(1,3) y residuos de xilosa β(1,2), a diferencia de los N-glicanos complejos de mamíferos que no presentan residuos de xilosa y presentan residuos de fucosa en enlace α(1,6). Además es típica de mamíferos la presencia de galactosa y ácido siáliaco. *Figura adaptada de Raikhel y Chrispeels (2000).*

En insectos la ruta de N-glicosilación es similar a la ya descrita en plantas hasta la acción de la α-Man II y GnT II (Figura IV). Una vez eliminados los residuos de manosa y añadido el residuo de N-acetilglucosanima, se produce la unión de un residuo de fucosa en enlace α(1,6) sobre la N-acetilglucosamina proximal del "*core*" de quitobiosa, mediante la acción de la enzima α(1,6)-fucosiltransferasa (Figura IV). A continuación, se une otra fucosa en enlace α(1,3) mediante la acción de la enzima α(1,3)-fucosiltransferasa, obteniendo de esta manera N-glicanos difucosilados. La acción de GnT I es probablemente esencial, al igual que ocurre en plantas, para la actuación de las fucosiltransferasas, puesto que se ha descrito en insectos un procesamiento específico de los residuos terminales GlcNAc de los N-glicanos añadidos por la GnT I después de la fucosilacion mediante la acción de la enzima β-N-acetilhexosaminidasa. (Fabini y Wilson, 2001; Wilson, 2002) (Figura IV).

En insectos, el N-glicano más complejo encontrado hasta el momento es el de la glicoproteína del veneno de abeja, que contiene un brazo GalNAcβ1,4 (α1,3-Fuc) GlcNAc así como dos residuos de α(1, 3)- y α(1 ,6)-fucosa (Fabini y Wilson, 2001) (Figura IV).

Como puede observarse, en muchos sentidos los N- glicanos de invertebrados son más afines a los de las plantas que a los de mamíferos u otros vertebrados (Wilson, 2002).

Los estudios de la estructura de N-glicanos de *Drosophila melanogaster* indican la presencia tanto de estructuras oligomanosídicas y estructuras con residuos de α(1,6)-fucosa como de glicanos difucosilados (Fabini y Wilson, 2001) indicando que al igual que en plantas se produce cierta heterogeneidad en el proceso (Figura IV).

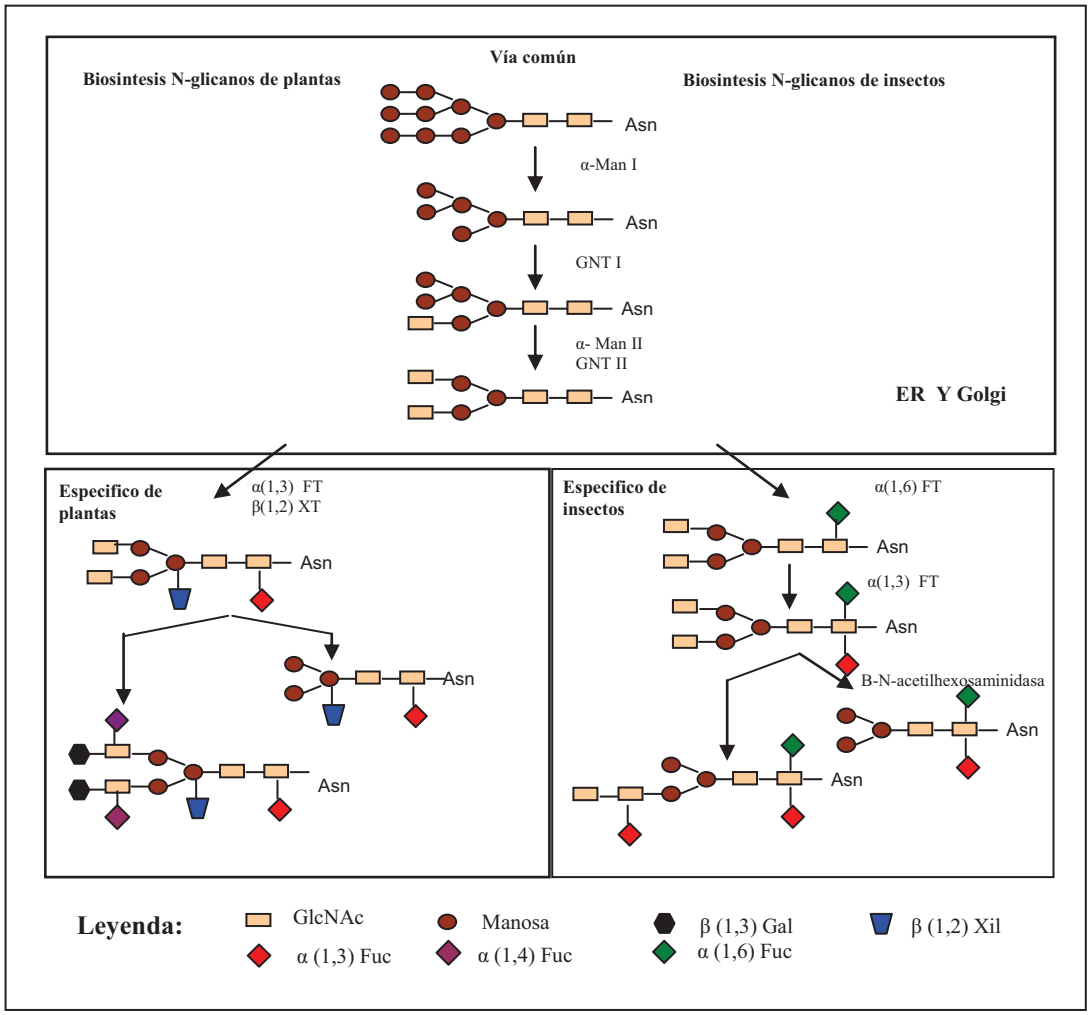


Figura IV. Biosíntesis de N-glicanos en plantas e insectos. La biosíntesis de los N-glicanos en plantas e insectos comienza con una vía común pero la acción de diferentes enzimas al final de la maduración de las proteínas origina N-glicanos diversos. Específico de plantas es la formación de los N-glicanos complejos por la actuación de las enzimas α(1,3)-FT y β(1,2)-XT que unen residuos de fucosa y residuos de xilosa. Posteriormente, pueden actuar las enzimas β(1,3)-GalT y α(1,6)-FT dando lugar a la denominada estructura de Lewis[a]. En insectos, se producen estructuras difucosiladas mediante la acción

de α(1,6)-FT y α(1,3)-FT. Se pueden eliminar residuos de GlcNAc terminales mediante la acción de la enzima β-N-acetilhexosaminidasa. *Figura adaptada de Wilson, 2002.*

En otro organismo modelo *Caenorhabditis elegans,* a pesar de su anatomía simple, la diversidad y complejidad de los N-glicanos existentes es sorprendente, especialmente cuando se compara con el patrón de N-glicosilación presente en *Drosophila melanogaster* (Paschinger y col., 2008). Esta variedad estructural se manifiesta en la presencia de oligosacáridos fucosilados y metilados, estructuras oligomanosídicas y N-glicanos con residuos α(1,6)-fucosa al igual que estructuras de oligosacáridos difucosilados típicas de insectos (Natsuka y col., 2002; Wilson, 2002).

4. Utilización de organismos fotosintéticos como biofactorías para la producción de proteínas recombinantes.

Las plantas transgénicas son consideradas uno de los sistemas más prometedores para la producción de proteínas recombinantes, tanto de interés terapéutico como agroalimentario e industrial (Chen y col., 2005). La utilización de plantas como factorías celulares para la producción de proteínas recombinantes (vacunas, anticuerpos, etc) es un área de investigación de creciente interés en los últimos años (Ma y col., 2003; León-Bañares y col., 2004; Ma y col., 2005). En la última década, más de 100 proteínas recombinantes han sido producidas en diferentes especies (Chen y col., 2005) Actualmente, hay disponibles una serie de sistemas de expresión para la producción de proteínas recombinantes y cada uno de estos sistemas ofrece limitaciones así como diferentes ventajas en términos de producción de proteína, facilidad de manipulación y coste de producción (Mayfield y col., 2003) (Tabla I).

Sistema	Coste	Tiempo	Capacidad de Re-escalar la producción	Procesamiento post-traduccional	Riesgo contaminación	Coste almacenamiento
Bacteria	Bajo	Corto	Alto	Ninguno	Endotoxinas	Intermedio
Levadura	Intermedio	Intermedio	Alto	Incorrecto	Bajo	Intermedio
Cultivo de células Animales	Alto	Largo	Muy bajo	Correcto	Virus,priones, ADN oncogénico	Alto
Animales Transgénicos	Alto	Muy largo	Bajo	Correcto	Virus,priones, ADN oncogénico	Alto
Cultivo de células vegetales	Intermedio	Intermedio	Intermedio	Pequeñas diferencias	Bajo	Intermedio
Plantas transgenicas	Muy bajo	Largo	Muy alto	Pequeñas diferencias	Bajo	Barato

Tabla I. Comparación entre los diferentes sistemas de producción de proteínas recombinantes. *Tabla adaptada del artículo de Ma y col., 2003.*

Las plantas presentan diversas características que las hacen más interesantes para la producción de proteínas recombinantes farmacéuticas comparadas con los sistemas tradicionales usados, como los cultivos celulares de insectos y de mamíferos y fermentación microbiana (Tabla I). Entre las ventajas más destacadas se incluyen el bajo coste de producción comparado con otros sistemas de expresión, rápida producción, ausencia de agentes patógenos humanos, capacidad de sintetizar y ensamblar proteínas glicosiladas complejas y la posibilidad de llevar a cabo otras modificaciones post-traduccionales (Ma y col., 2003; Chen y col., 2005).

La hormona de crecimiento humano fue la primera proteína recombinante con potencial terapéutico que fue expresada en plantas (Barta y col. 1986; Kang y col, 2009). Posteriormente, Hiatt y col. (1989) expresaron correctamente en tabaco el primer anticuerpo funcional producido en plantas transgénicas. Las proteínas actualmente producidas en plantas pueden ser categorizadas en 4 áreas: vacunas, anticuerpos monoclonales, proteínas biofarmacéuticas y proteínas de interés industrial (enzimas) (Tabla II).

Año	Proteína	Especies transformadas	Niveles de expresión	Referencia
VACUNAS:				
2003	Antigeno hepatitis B (HbsAg)	Tomate Cherry	300mg hoja PF 10ng/g fruto PF	Gao y col., 2003.
2002	Toxina B	Semilla de maíz	10% TSP/0.1% PF	Streatfield y col., 2003.
2002		Tabaco	8% TSP,1%PF	Silva y col., 2002.
2002	Toxina B del cólera humano	Tomate	0.04%TSP,0.005%PF	Jani y col., 2002.
2001	Toxina B del colera	Cloroplasto de tabaco	4.1%TSP	Daniell y col., 2001.
2000 **1999**	Antigeno hepatitis B (HBsAG)	Patata, Lechuga	1.1µg-16 µg/g tubérculo fresco, 0.01%PF	Richter y col., 2000. Kapusta y col., 1999.
ANTICUERPOS:				
2003,2002	Proteina VP6 de rotravirus grupo A	Patata	0.01%TSP, 0.006%-0.002%TSP	Yu y col., 2003. Matsumura y col., 2002.
2002		Arabidopsis	-	Bouquin y col., 2002.
2000	ScFvT84. 66 (ScFV)	Trigo	0.9 µg/g hojas, 1.5 µg/g semillas	Staub y col., 2000.
1999	Anticuerpo C5-1	Alfalfa	0.31 y 1.0% TSP	Khoudi y col., 1999.
1998	HSV-2	Soja	-	Zeitlin y col., 1998.

1995	Guy´s13 (sIgA)	Tabaco	500 µg/g PF	Ma y col., 1995. Ma y col., 1998.
PROTEINAS BIOFARMACEUTICAS:				
2003	Avidina	Maiz	20%TSP	Masarik y col., 2003.
2002	Lactoferrina humana	Arroz	0.5% pesos seco	Nandi y col., 2002.
2000	Somatotropina humana	Cloroplasto de tabaco	7.00%TSP	Leite y col., 2000
1999	Aprotinina	Maiz	-	Zhong y col., 1999. Lee y col., 1997.
1996	Glucocerebrosidasa	Tabaco	1.00%-10.00%TSP	Cramer y col., 1996
1995	Hirudina	Col	3% proteínas de semilla	Parmenter y col., 1995.
1995	Eritrogenina	Tabaco	0.01%TSP	Fischer y col., 2000.
1994	Alfa-tricosantina	Virus mosaico de tabaco	2.00%TSP	Kumagai y col., 1993.
1993	Factor de crecimiento epidérmico humano	Tabaco	0.01%TSP	Fischer y col., 2000.
1990	Albúmina de suero humano	Tabaco	0.02%TSP	Sijmons y col., 1990.
ENZIMAS INDUSTRIALES:				
2004	Lacasse	Maiz	0.2%TSP	Bailey y col., 2004.
2003	Lacasse	Maiz	0.1%-0.5%TSP	Hood y col., 2003.

Tabla II. Proteínas producidas en plantas transgénicas. PF: peso fresco; TSP: proteína soluble total. *Tabla adaptado del artículo de Chen y col., 2005.*

A pesar de las ventajas descritas, existen algunos problemas como los bajos niveles de expresión, problemas de estabilidad de las proteínas recombinantes y presencia de estructuras glicosídicas no deseables por su diferencia con las humanas (Daniell y col., 2005).

Una alternativa a estos problemas consiste en expresar proteínas recombinantes en el cloroplasto o destinar la proteína de interés a dicho orgánulo (Daniell y col., 2005; Chen y col., 2005) La transformación cloroplástica para la obtención de proteínas recombinantes presenta una serie de ventajas frente a la transformación nuclear, como son: la bioseguridad (al tener confinado el transgen en el cloroplasto cuya herencia materna restringe su posible liberación al ambiente), la existencia de múltiples cloroplastos en cada célula con múltiples copias del genoma plastídico lo que posibilita un alto nivel de expresión de las proteína recombinantes y la posibilidad de co-expresar

múltiples genes en operones lo que resulta conveniente para las vacunas que requieren múltiples epítopos para su correcto funcionamiento. (Lössl y col, 2011) Además los cloroplastos pueden procesar proteínas eucariotas, incluyendo el plegamiento correcto y la formación de puentes disulfuro. Las proteínas se pueden acumular hasta aproximadamente un 40 % de las proteínas solubles totales.

Más de la mitad de las proteínas humanas son glicoproteínas y su función depende con frecuencia de sus glicanos, que afectan a su vida media en plasma, la actividad del tejido objetivo y/o a su actividad biológica. De manera parecida, más de un tercio de las proteínas biofarmacéuticas aprobadas son glicoproteínas. Muchas proteínas terapéuticas (como anticuerpos, vacunas, factores de sangre y los interferones) son glicoproteínas (Walsh y Jefferies, 2006; Saint-jore-dupas y col., 2007; Gomord y col., 2010) y tanto su función como su eficiencia se ven afectados por la presencia y composición de sus N-glicanos.

La N-glicosilación de proteínas es esencial por diversas razones que incluyen la estabilidad, solubilidad, plegamiento y actividad biológica (Lerouge y col., 1998, Chen y col., 2005). Como ya hemos mencionado, la glicosilación de las proteínas en plantas y animales difiere en detalles concretos. Pequeños cambios en la estructura de un glicano conllevan cambios drásticos en la actividad, biodistribución y tiempo de vida de la proteína recombinante con respecto a la nativa. Las proteínas recombinantes derivadas de plantas presentan residuos β(1,2)-xilosa y α(1,3)-fucosa y además carecen de residuos de galactosa terminal en enlace β(1,4) y de ácido siálico característicos de las glicoproteínas de humanos (Bakker y col., 2001b; Ma y col., 2003).Además, el suero humano contiene anticuerpos que reaccionan contra los residuos de β(1,2)-xilosa y α(1,3)-fucosa provocando respuestas alérgicas (Lerouge y col., 2000; Bardor y col., 2003; Ma y col. 2003; Chen y col., 2005).

Por todo ello, es importante conocer en detalle el proceso de glicosilación en plantas, para elaborar estrategias encaminadas a la modificación genética de las plantas con el fin de producir proteínas con glicosilación específica de mamíferos.

En los últimos años se ha hecho un gran esfuerzo en la síntesis de proteínas recombinantes en plantas y se han llevado a cabo diferentes estrategias para la humanización de las glicoproteínas. El proceso de humanización de glicoproteínas expresadas en plantas requiere la eliminación o prevención de la adición de los residuos de fucosa y xilosa y la adición de galactosa y ácido siálico. La primera estrategia es la retención de la glicoproteína recombinante en el retículo endoplásmico, de manera que se pueda evitar una mayor modificación de las glicoproteínas en el aparato de Golgi. La segunda es modificar la maquinaria enzimática del aparato del Golgi mediante el silenciamiento de enzimas o genes relacionados con xilosa y fucosa o por adición de nuevas glicosiltransfersas para modificar los N-glicanos.

Las proteínas que residen en el lumen del RE de plantas contienen N-glicanos ricos en manosa con estructura común a la de mamíferos. En plantas, como en otras células eucarióticas, la retención de las proteínas solubles en el RE está basada en la presencia en el C-terminal de la señal de retención: KDEL o HDEL. La adición de la secuencia H/KDEL en el C-terminal de una proteína es suficiente para retenerla en el RE de plantas (Gomord y col., 1997). Recientemente se ha demostrado en plantas de tabaco que los anticuerpos expresados con una secuencia KDEL fusionada en el extremo C-terminal de las cadenas pesadas y ligeras contienen exclusivamente N-glicanos ricos en manosa (Petruccelli y col., 2006; Saint-jore-dupas y col., 2007).

Estrategias como el uso de β(1,4)-galactosiltransferasa humana y sialiltransferasas purificadas para modificar las proteínas recombinantes *in vitro*, así como la expresión del gen que codifica la β(1,4)-galactosiltransferasa en plantas transgénicas, servirían para producir anticuerpos recombinantes con un N-glicano similar al de humanos (Bakker y col., 2001b). Se ha demostrado que la enzima β(1,4)-galactosiltransferasa puede expresarse en plantas de tabaco, que son capaces de humanizar parcialmente glicanos de glicoproteínas endógenas, así como de expresar transgénicamente anticuerpos de mamíferos (Bakker y col., 2001b).

Por otro lado, se han diseñado estrategias para la producción de proteínas recombinantes con N-glicanos similares a los de mamíferos usando plantas con modificaciones en el mecanismo enzimático para la maduración de los glicanos mediante desactivación de enzimas especificas del Golgi o creando plantas mutantes que tenga afectado el proceso de biosíntesis del N-glicano (Lerouge y col., 2000).

Existen mutantes que facilitan estrategias para formar proteínas recombinantes con N-glicanos parecidos a los de mamíferos. El mutante *cgl1* de *Arabidopsis* carece de actividad GnT I y por tanto no incorpora residuos de xilosa ni fucosa por lo que no se sintetizan glicanos complejos (Strasser y col., 2004). Otro mutante en *Arabidopsis thaliana,* deficiente en la actividad β(1,2)-xilosiltransferasa y las dos α(1,3)-fucosiltransferasas (Strasser y col., 2004), genera plantas que pueden producir N-glicanos con dos residuos de N-acetilglucosamina terminales y que no contienen residuos ni de xilosa ni de fucosa. En el 2003, β(1,2)-xilosiltransferasa y α(1,3)-fucosiltransferasa fueron silenciadas por recombinación homóloga en *Physcomitrella patens* (Koprivova y col. 2004*.)* No obstante, este tipo de estrategia es complicada ya que exige la generación de dobles y triples mutantes, con todo lo que esto implica. Como alternativa, se ha establecido el silenciamiento de genes mediante RNAi de la β(1,2) xilosil y α(1,3) fucosiltransferasa en diferentes especies de plantas como *Nicotiana benthamiana*, *Medicago sativa* y L*emna minor* (Bosch y col., 2010).

El descubrimiento ya mencionado anteriormente de Villarejo y colaboradores en 2005 de la ruta de síntesis de glicoproteínas a través del sistema de endomembranas para su transporte al cloroplasto en *Arabidopsis,* adquiere relevancia como potencial herramienta para aumentar y mejorar el rendimiento en la expresión de glicoproteínas

recombinantes en plantas. La producción de glicoproteínas de interés, mediante sistemas que permiten controlar el destino, la estabilidad y la bioseguridad del producto final es uno de los mayores retos de los proyectos de biotecnología en la actualidad. La expresión de proteínas recombinantes en el cloroplasto permitiría aumentar no sólo los rendimientos en cantidad de proteína, sino también su facilidad de aislamiento.

5. Chlamydomonas reinhardtii.

5.1 Clasificación, morfología y ciclo de vida.

Chlamydomonas reinhardtii es un alga verde unicelular ampliamente utilizada como organismo fotosintético modelo, en estudios tanto genéticos como bioquímicos y de biología celular, debido entre otros factores a su facilidad de cultivo y la disponibilidad de una serie de herramientas para su manipulación y estudio.

C.reinhardtii pertenece a la división Chlorophyta, clase Chlorophyceae, orden Volvocales, familia Chlamydomonadacea, género Chlamydomonas (Strasburger y col., 1994).

Clásicamente, las especies de *Chlamydomonas* se han definido basándose únicamente en criterios morfológicos. Se distinguen de otras Volvocales unicelulares por la presencia de pared celular, de un par de flagelos apicales y de un cloroplasto que rodea uno o más pirenoides (Harris, 1989; 2001).

El género *Chlamydomonas* se encuentra ampliamente distribuido por todo el planeta y las distintas especies ocupan nichos ecológicos que van desde el suelo (de donde procede la cepa de laboratorio *C.reinhardtii*) a los glaciares (*C. navalis*), las minas ácidas o las turbas y aguas residuales.

Dentro del género, los parámetros de identificación morfológicas son el tamaño y forma celular, la forma y posición del cloroplasto y el/los pirenoides, la longitud de los flagelos y otras características estructurales más sutiles (Harris, 2001).

Se han descrito más de 500 especies de *Chlamydomonas* aunque científicamente sólo se trabaja con unas cuantas especies, siendo *C. reinhardtii* la especie predominante y más empleada en el laboratorio por su facilidad de cultivo en condiciones controladas de laboratorio, la capacidad de utilizar acetato como única fuente de carbono sin necesidad de realizar fotosíntesis y un ciclo celular simple y bien definido. Todas estas características y otras, han convertido a *Chlamydomonas* en un organismo modelo ampliamente utilizado.

Se piensa que las principales cepas de *C.reinhardtii* derivan de la aislada por GM Smith en 1945 a partir de una muestra de suelo recolectada cerca de Amherst, Massachusetts. (Harris, 2001; Proschold y col., 2005).

Las células de *Chlamydomonas* se caracterizan por su forma esférica-ovalada con un tamaño que varía entre las 8 y las 22 µm de diámetro según el momento del ciclo en el que se encuentre. Posee un par de flagelos en la zona apical de la célula que terminan en el interior celular en un par de cuerpos basales conectados por fibras estriadas. Generalmente, se pueden apreciar dos vacuolas contráctiles entre los cuerpos basales y el núcleo. El núcleo se encuentra en gran parte rodeado por un único cloroplasto en forma de copa que ocupa los dos tercios de la parte basal de la célula y en el que se sitúa el pirenoide (Harris, 1989; 2001). El pirenoide es un estructura proteica electrodensa presente en el cloroplasto de la mayoría de las algas. En él se lleva a cabo la fijación de CO_2 y puede acumular hasta el 90% de la RuBisCo de la célula (Borkhsenious y col., 1998). Justo bajo la membrana del cloroplasto, en el ecuador de la célula, se encuentra el "eyespot" o estigma, un primitivo aparato visual, de color naranja intenso debido a la alta concentración de carotenoides que sirve a la célula para detectar la dirección e intensidad de la luz (Kreimer, 2009). La mitocondria es mucho más pequeña que el cloroplasto, ocupando alrededor de un 3% del volumen de la célula, se encuentra dispersa por el citosol y en las secciones de la célula puede aparecer como pequeños cuerpos ovalados o alargados (Bennoun y col., 1995) (Figura V).

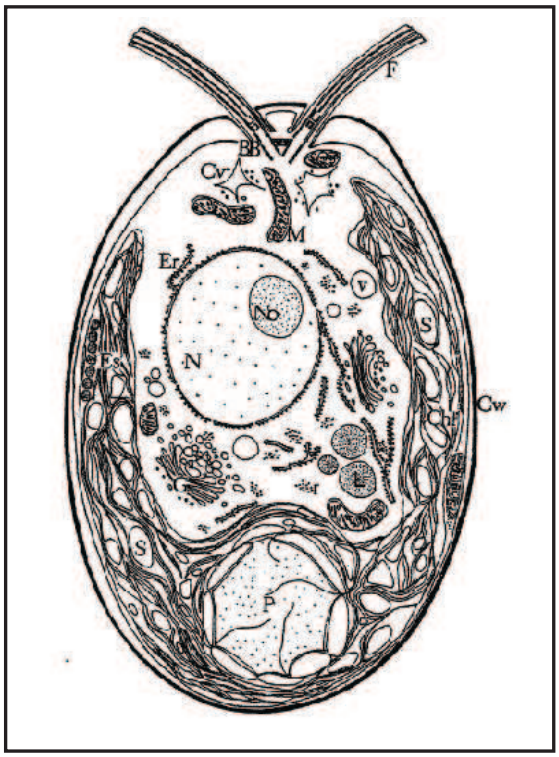


Figura V. Representación de una célula de Chlamydomonas en interfase. Tamaño 10 µm; BB: cuerpos basales; Ch: cloroplasto; Cv: vacuolas contráctiles; Cw: pared celular; Er: retículo endoplásmico; Es: "eyespot" o estigma; F: flagelo; G: aparato de Golgi; L: Cuerpo lipídico; Mi: mitocondria; N: núcleo; No: nucléolo; P: pirenoide; r: ribosomas; S: granulosde almidón d; V: vacuola. *Imagen Harris ,2001.*

C. reinhardtii es un organismo eucariota de rápido crecimiento y fácil cultivo que presenta un ciclo biológico haplo-diplonte. Se reproduce normalmente de forma asexual, por mitosis, generando nuevas células vegetativas haploides (Harris, 2001). Estas células vegetativas pueden ser de sexo (*mating type*) positivo (mt+) o negativo

(mt-). Esta distinción no tiene importancia en el proceso normal de reproducción asexual. En crecimiento logarítmico en un ciclo de luz:oscuridad de 12:12 h, las células permanecen en G1 durante el periodo de luz y se dividen sincrónicamente durante el periodo de oscuridad, generalmente en dos divisiones mitóticas sucesivas, quedando las cuatro células hijas dentro de una pared celular común de la que se liberarán simultáneamente al secretarse una enzima lítica (Spessert y Waffenschmidt, 1990). Sin embargo, en condiciones de estrés, como puede ser la limitación de nutrientes (como el nitrógeno del medio) o cambios en las condiciones de luz (luz azul), *C. reinhardtii* se reproduce sexualmente, induciéndose la transformación de las células vegetativas en gametos sexualmente competentes (Grossman, 2000; Harris, 2001). Los gametos de sexo opuesto se aparean y se fusionan, dando lugar a zigotos diploides (Grossman, 2000; Harris, 2001). Dichos zigotos pueden permanecer meses en estado latente, germinando cuando las condiciones ambientales son favorables y dando lugar, tras un proceso de meiosis a veces seguido de una mitosis adicional, a células vegetativas haploides. También existe la posibilidad de que los zigotos no puedan germinar y se transformen en células vegetativas diploides estables, capaces de dividirse por mitosis (Harris, 1989) (Figura VI).

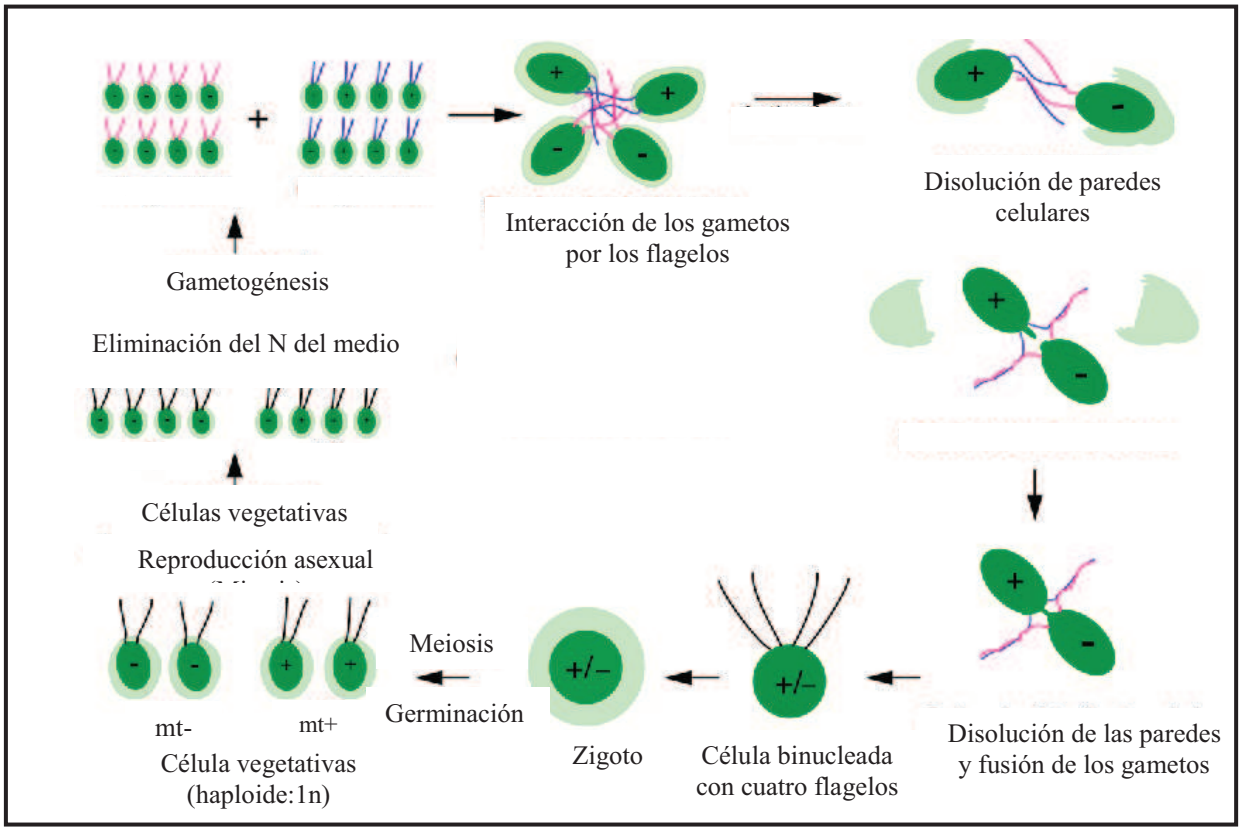


Figura VI: Ciclo de vida del alga verde Chlamydomonas reinahrdtii. *Imagen adaptada del articulo de Harris, 2001.*

5.2 Chlamydomonas reinhardtii la *"levadura verde".*

C. reinhardtii se ha denominado la levadura verde para enfatizar todas las ventajas técnicas de su uso como organismo modelo (Goodenough, 1992; Rochaix, 1995).

Las células de *C. reinhardtii* pueden cultivarse tanto en medio liquido como en medio solidificado con agar, a pH neutro y sin necesidad de añadir vitaminas ni cofactores (Harris, 1989). Tiene por tanto la ventaja de crecimiento microbiano, con tiempos de duplicación en condiciones óptimas menores de 10 horas, sin la complejidad de la multicelularidad.

Además, *C.reinhardtii* puede crecerse fotoautotrófica, mixotrófica o heterotróficamente, lo que ha permitido la generación y el estudio de numerosos mutantes fotosintéticos (Nickelsen y Kuck, 2000). Al poder utilizar acetato como fuente de carbono tanto en oscuridad como en luz, los mutantes afectados en fotosíntesis son viables si se les suministra acetato en el medio (Grossman, 2000; Dent y col., 2001).

Tradicionalmente se ha empleado *Chlamydomonas reinhardtii* para el estudio de la estructura y ensamblaje del flagelo, gametogénesis, fototaxis y respuesta fisiológica a la luz y a los nutrientes, entre otros procesos (Harris, 2001). Las actuales herramientas moleculares aplicadas a este organismo, junto con las técnicas de análisis genético y la existencia de numerosos mutantes fotosintéticos han hecho de esta alga una poderosa herramienta para el estudio de la regulación de la expresión génica en el cloroplasto.

Otra ventaja de *C.reinhadtii* es que su genoma se encuentra secuenciado (Merchant y col., 2007). Una de las principales características del genoma de este organismo es su elevado contenido en G+C, de un 64%, llegando a alcanzar el 68% en las regiones codificantes, así como la alta densidad de secuencias de ADN repetitivo (Merchant y col., 2007).

La facilidad de control de su ciclo sexual permite el análisis de tétradas, lo que la convierte en un buen modelo para estudios genéticos. Además, es unos de los pocos organismos en el que sus tres genomas (mitocondrial, cloroplástico y nuclear) pueden ser transformados (Harris, 2001). La transformación de los genomas del cloroplasto y la mitocondria se realiza mediante métodos biolísticos (bombardeo de partículas) (Ramesh y col., 2004; Remacle y col., 2006). La transformación del genoma nuclear se puede llevar a cabo fácilmente mediante la agitación vigorosa de las células de *C.reinhardtii* y el ADN exógeno en presencia de perlas de vidrio (Kindle, 1998). Sin embargo, al contrario de lo que ocurre en la transformación del genoma del cloroplasto, donde el ADN exógeno se incorpora por recombinación homóloga, en la transformación del genoma del núcleo la incorporación de ADN recombinante es al azar y genera con cierta frecuencia delecciones de ADN en la región de inserción, lo que complica el análisis de los mutantes generados (Tam y Lefrebvre, 1993).

Ante la imposibilidad efectiva de realizar mutaciones dirigidas en genes del genoma nuclear de *C.reinhardtii,* se han desarrollado técnicas para disminuir la expresión de genes específicos mediante el silenciamiento de ARN. Para ello se han utilizado con éxito construcciones que expresan ARNs antisentido (Schoda y col., 1999), ARNs con repeticiones invertidas (Fuhrmann y col., 2001) o micro ARN artificiales (Zhao y col., 2009; Molnar y col., 2009) y se sigue trabajando para mejorar la eficiencia de estas técnicas en *C.reinhardtii* (Schoda, 2006).

5.3 Chlamydomonas reinhardtii como herramienta emergente en la producción de proteínas recombinantes

Actualmente se trabaja activamente en la generación de algas transgénicas para la expresión de proteínas recombinantes. Como se ha mencionado anteriormente, las algas verdes han servido como organismos modelo para el estudio de procesos biológicos básicos, pero estos organismos habían recibido menos atención en lo que respecta a su utilización como fábricas celulares para la producción de proteínas recombinantes (Franklin y Mayfield, 2004).

C. reinhardtii posee algunas características que aumentan enormemente su interés para la producción de proteínas recombinantes como son: la facilidad de transformación de su ADN nuclear y cloroplástico, la capacidad y versatilidad de crecimiento de sus cultivos, ya que pueden crecer en escalas desde pocos mililitros a 500.000 litros en fermentadores de manera efectiva, reduciendo la posibilidad de contaminación y con bajos costes (Mayfield y col., 2007; Rasala y col., 2010; Specht y col., 2010). Además, como se ha mencionado anteriormente, pueden crecer heterotróficamente o fototróficamente. Comparada con plantas, *C. reinhardtii* crece mucho más rápido, doblando el número de células en aproximadamente 8 horas. Por otro lado, como se propaga por división vegetativa, el tiempo desde la transformación inicial hasta la producción de proteínas es significativamente menor que en plantas, requiriendo tan solo 6 semanas para la producción de proteínas en matraces (Figura VII). Otra ventaja es la facilidad de purificación de las proteínas recombinantes producidas en el alga con respecto a las obtenidas en plantas (Mayfield y col., 2005; 2007).

Estos atributos y el hecho de que a las algas verdes se las incluye en la categoría GRAS (*"generally regarded as safe")* hace de *C. reinhardtii* un sistema muy atractivo para la producción de proteínas recombinantes y una alternativa al sistema de plantas (Franklin y Mayfield, 2004; Rosenberg y col., 2008). En la actualidad se están desarrollando numerosas herramientas para *Chlamydomonas reinhardtii* que la convierten en una prometedora plataforma para la expresión de proteínas recombinantes. Se han desarrollado técnicas que permiten una expresión robusta tanto en el genoma nuclear como el plastídico. Con todas estas ventajas, diferentes grupos de investigación han considerado el empleo de esta alga y otras microalgas como maquinas

biológicas capaces de producir péptidos y proteínas recombinantes (Specht y col., 2010).

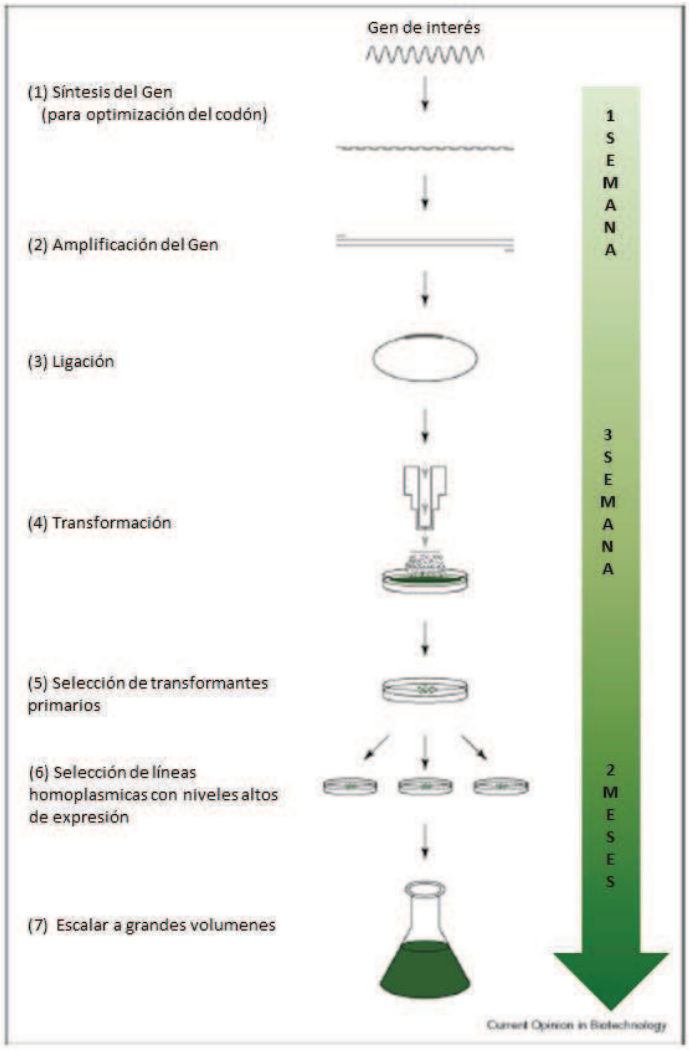


Figura VII. Etapas para la producción de proteínas recombinantes en C.reinhardtii. *Imagen adaptada del artículo de Mayfield y col., 2007.*

Recientemente ha surgido un interés especial en la producción de proteínas recombinantes de interés medico en el cloroplasto de *C.reinhardtii*, en el que ya se han producido varias de estas proteínas.

Con respecto a proteínas recombinantes de interés médico a nivel nuclear en esta microalga, sólo se conoce la producción de Eritropoyetina humana, con un rendimiento aproximado de 100 microgramos por litro de cultivo. (Eichler-Stahlberg y col., 2009). Conviene recordar, como se ha mencionado más arriba, que el mecanismo de inserción del transgen en el núcleo se da al azar, lo que incrementa la posibilidad de silenciamiento génico, a diferencia del cloroplasto, donde la inserción se da de manera dirigida en un sitio preciso mediante un proceso de recombinación homóloga.

En la Tabla III se presenta la información de diversas proteínas de interés médico producidas hasta el momento en el cloroplasto de *Chlamydomonas reinhardtii*.

Cabe mencionar que se ha logrado la producción de anticuerpos monoclonales y como ejemplo de ello tenemos el trabajo realizado por Mayfield y col. (2003), donde se logró exitosamente la producción del anticuerpo monoclonal denominado HSV8-lsc, el cual presenta actividad contra la glucoproteína D del virus del herpes simple. En este trabajo se demostró la capacidad del cloroplasto de esta microalga para formar correctamente puentes disulfuro en el interior de la estructura proteica, que son de gran relevancia para la producción de proteínas recombinantes. Tran y col. (2009), lograron ensamblar el anticuerpo monoclonal 83K7C (derivado de la IgG1 humana), que tiene aplicación contra el ántrax y que demostró una actividad similar a los anticuerpos obtenidos en mamíferos. Estos mismos autores produjeron tres anticuerpos monoclonales más en el cloroplasto de *C.reinhardtii*, confirmando su capacidad como biorreactor para producir este tipo de anticuerpos, semejantes a aquellos producidos en otros sistemas eucariontes.

Se ha demostrado también la capacidad de *C.reinhardtii* para producir vacunas recombinantes. Una vacuna contra la infección de la bacteria *Staphylococcus aureus* fue producida por Dressen y col., (2010) en el cloroplasto de esta microalga, la cual fue capaz de provocar inmunidad por vía oral en ratones. También en 2010, Dauvillée y colaboradores desarrollaron gránulos de almidón confinados en el cloroplasto de *C.reinhardtii* genéticamente modificados con distintos candidatos antigénicos de distintas especies de *Plasmodium.* Estos gránulos de almidón aportados en la dieta resultaron ser capaces de inmunizar ratones frente a la malaria.

En el cloroplasto de *C.reinhardtii* se han producido también algunas proteinas recombinantes terapeuticas. Un ejemplo es un suero amiloide asociado a las glandulas mamarias (M-SAA), producido por Manuell y col. (2007); esta proteina se encuentra de manera natural en el calostro bovino, y es capaz de estimular las celulas epiteliales humanas para producir mucina intestinal, la cual actua como agente profiláctico contra infecciones virales y bacterianas. Otro ejemplo es la enzima acido glutámico descarboxilasa (GAD65) producida por Wang y col. (2008), esta enzima puede ser usada como autoantígeno, ya que muchos de los pacientes que presentan diabetes tipo I de reciente aparición presentan anticuerpos contra GAD65, los cuales pueden servir para predecir y dar un diagnostico temprano de la diabetes tipo I, enfermedad resultante de la destruccion de las celulas productoras de insulina en el pancreas.

Rasala y col. (2010) evaluaron la produccion de siete proteínas que son o presentan potencial como agentes terapéuticos humanos. Entre las proteínas evaluadas, se encuentran la eritropoyetina (regulador de la sintesis de eritrocitos), las proteinas 10FN3 y 14FN3 (dominios de la fibronectina implicados en la adhesion celular), el interderon humano beta-1 (mejora la integridad de la barrera hematoencefalica), la proinsulina humana (hormona que reguala los niveles de azucar en sangre), el factor de creicimiento endotelial vascular humano (VEGF; que estimula la angiogénesis) y la proteina B1 del grupo de alta movilidad (HMGB1; mediadora en la curacion de heridas mediante la activacion de celulas endoteliales).

Gen expresado	Tipo de proteína	Función	Promotor + 5´UTR/3´UTR	Rendimiento (% proteína soluble total, %PST)	Aplicación	Referencia
HSV8-lsc	Anticuerpo	Actividad contra la glicoproteína D del virus herpes simple	atpA/rbcL	0.5% PST	Anticuerpos monoclonales	Mayfield y col., 2003
83K7C	Anticuerpo derivado de IgG1	Actividad contra el antígeno PA83 del ántrax	LC: psbA/rbcL HC: psbA/psbA	0.1% de biomasa seca	Anticuerpos monoclonales	Tran y col., 2009
CTB-D2	Toxina B del cólera fusionada con el dominio D2 de la proteina de unión a la fibronectina	Vacuna oral contra *S. aureus*	rbcL/rbcL	0.7% PST	Vacuna Oral	Dressen y col., 2010
Htrail	Factor de necrosis tumoral	Tratamiento del cáncer	atpA/rbcL	0.43%-0.67% PST	Terapéutico	Yang y col.,2006
M-SAA	Suero bovino amiloide asociado a las glándulas mamarias	Estimulación de la producción de mucina en el instestino para inmunizar a recién nacidos	psbA/psbA	5.0% PST	Terapéutico	Manuell y col.,2007
hGAD65	Ácido glutámico descarboxilasa humana 65	Diagnóstico temprano de la diabetes tipo I	rbcL/rbcL	0.25-0.3% PST	Terapéutico	Wang y col.,2008
M-SAA-10NF3	Dominio 10 de la fibronectina III humana	Símil de anticuerpo	psbA/psbA	-3.0% PST	Terapéutico	Rasala y col.,2010
14NF3	Dominio 14 de la fibronectina III humana	Símil de anticuerpo	psbA/psbA	3% PST	Terapéutico	Rasala y col.,2010
VEFG	Factor de crecimiento endotelial vascular humano	Tratamiento del efisema pulmonar y de la disfunción eréctil; antidepresivo	psbA/psbA	2% PST	Terapéutico	Rasala y col.,2010
HMGB1	Proteína B1 del grupo de alta movilidad	Curación de heridas mediante la activación de células endoteliales	psbA/psbA	2.5% PST	Terapéutico	Rasala y col.,2010

Tabla III: Proteínas recombinantes de uso médico obtenidas por transformación cloroplástica en Chlamydomonas reinhardtii. Resumen de las proteinas recombinantes que han sido producidas en el cloroplasto de *C.reinhardtii*, incluyendo sus rendimientos proteicos, asi como los elementos reguladores de la expresion genica (promotor + 5´UTR/3´UTR) con los que se ha logrado dicha producción.

Cuatro de las proteinas evaluadas (10FN3,14FN3,VEGF y HMGB1) fueron producidas con éxito, ya que se obtuvieron rendimientos de entre 2 y 3 por ciento de proteina soluble total en el cloroplasto de *C.reinhardtii*. Este trabajo demuestra que la expresión de proteínas recombinantes en los cloroplastos de algas está a la par con otras

plataformas de expresión, y muestra que la expresión de proteínas complejas en el cloroplasto de algas es tan viable como en cualquier sistema de eucariotas (Specht y col., 2010).

A pesar del atractivo de *C. reinhardtii* para su utilización como biofactoría de proteínas recombinantes, aún se desconoce en este organismo las características generales del proceso de N-glicosilación de proteínas y la existencia de una ruta similar a la descrita en *Arabidopsis*, de transporte de N-glicoproteínas al cloroplasto.

La expresión de glicoproteínas heterólogas en el cloroplasto de las algas verdes permitiría superar algunas dificultades (como bajo rendimiento de producción y baja estabilidad de la molécula producida) e incrementar el rendimiento. Sin embargo, hasta ahora no existía ningún estudio que demostrara la presencia de una ruta similar en *Chlamydomonas* y/o otras algas verdes a la encontrada en *Arabidopsis thaliana* (Villarejo y col, 2005). La conjunción en un mismo organismo de una vía de N-glicosilación de proteínas simple y de una ruta que permitiese acumular glicoproteínas en el cloroplasto, haría de este organismo el ideal para ser usado como factoría para la producción de glicoproteínas recombinantes.

II. OBJECTIVES

II. Objectives

The final aim of our study would be the use of *Chlamydomonas reinhardtii* as biofactory for the production of heterologous glycoproteins of interest to be directed to the chloroplast, as organelle that allows improving the efficiency of the production process of these glycoproteins.

For that, we must confirm several aspects still not established in this organism, as it is the existence of the pathway for targeting N-glycoproteins to the chloroplast in *Chlamydomonas reinhardtii* through the endomembrane system, as described in higher plants and in the other hand, to characterize the pathway of N-glycan biosynthesis as well as and the N-glycan composition and structure in this organism.

Based on these budgets, the specific goals of this work are:

- To find out if *Chlamydomonas reinhardtii* present the pathway to target N-glycoproteins to the chloroplast through the endomembrane system (ER and Golgi). For this purpose we should confirm the presence of glycoproteins in pure fractions of chloroplasts isolated from *Chlamydomonas* and if so, if these plastid N-glycoproteins reach the chloroplasts when transport through ER-Golgi system is blocked or disturbed at different stages.

- To characterize the biosynthetic pathway of N-glycosylation of proteins in *Chlamydomonas reinhardtii*. For that, we need first to perform an *in silico* analysis of orthologous enzymes involved in the N-glycosylation pathway in other organisms. The identification of the genes coding for these enzymes would allow us to characterize the route of N-glycosylation of proteins in *Chlamydomonas* and to facilitate the study of the N-glycan structure and composition.

- To analyse the structure and composition of the N-glycan linked to endogenous algal glycoproteins. We designed a strategy based on the use of different approaches: Biochemical, immunological, genetic and analysis by mass spectrometry (MALDI-TOF).

It is important to mention that the present work has been developed on the frame of a European project entitled: “A new scenario for the production of recombinant proteins in algae. Exploiting a native pathway for targeting glycoproteins to the algal chloroplast”, funded by the Plant-KBBE program, whose members include, in addition to the Spanish group from the Universidad Autónoma de Madrid, a French group, expert in Glycobiology, leading by Dr. Muriel Bardor, from the University of Rouen and a German group, expert in *Chlamydomonas* chloroplast Proteomics, leading by Prof. Michael Hippler from the University of Muenster.

III. MATERIALES Y MÉTODOS

III. Materiales y Métodos

1 Materiales.

1.1 Material biológico.

Para realizar este trabajo se utilizaron:

- Diferentes cepas de *Chlamydomonas reinhardtii*:

- La cepa de *Chlamydomonas reinhardtii* cc-503 cw 92 (Duke University, Durham, NC USA (Chlamydomonas Culture Collection)), es un mutante sin pared celular, que se utiliza como estirpe silvestre en numerosos laboratorios.

- La cepa de *Chlamydomonas reinhardtii* cc-425 arg2 cw15 (Duke University, Durham, NC USA (Chlamydomonas Culture Collection)), es un mutante auxotrófico para arginina empleado para transformación nuclear.

- La cepa de *Chlamydomonas reinhardtii* cc-1036 (Duke University, Durham, NC USA (Chlamydomonas Culture Collection)) es una estirpe mutante totalmente inmóvil que presenta un defecto que ocasiona rigidez en los flagelos. Posee pared celular. Cepa parental a partir de la cual se obtuvo el mutante L23 (Bloodgood y col., 1987).

- La cepa de *Chlamydomonas reinhardtii* L23, es un mutante cuyas glicoproteínas flagelares carecen de un epitopo glucídico reconocido por el anticuerpo FMG-1. Fue cedido por el Dr. Robert A. Bloodgood de la Universidad de Virginia (Estados Unidos) (Bloodgood y col., 1987).

- Tres líneas de *Arabidopsis thaliana:*

- Plantas de *Arabidopsis thaliana* silvestre ecotipo Columbia 0+.

- El mutante *cgl1* de *Arabidopsis thaliana* el cual carece de actividad GnTI (N-acetilglucosaminiltransferasa I) y como consecuencia acumula estructuras oligomanosídicas y carece de glicanos complejos (von Schaewen y col., 1993).

- El doble mutante FT11/12 de *Arabidopsis thaliana* en el cual los dos genes que codifican para la α(1,3)-fucosiltransferasa (*ft11* (At3g19280) y *ft12* (At1g49710)) (Strasser y col., 2004) han sido interrumpidos por inserción de T-DNA, provocando que las glicoproteínas del mismo carezcan de fucosa en la posición α(1,3) (Forth y col., datos no publicados).

1.2 Medios y condiciones de cultivo.

1.2.1 Medios de cultivo.

Las diferentes estirpes de *Chlamydomonas* se conservaban en medio TAP (Tris-Acetato-Fosfato) (Harris, 1989) (Tabla IV) solidificado con agar 1.5 % que se esterilizaba en autoclave a 110 ºC y 1 atm de presión durante 15 min. Este medio contiene ácido acético, que permite crecer a *Chlamydomonas* heterotróficamente usando acetato como fuente de carbono (Harris, 2001).

COMPOSICION	CONCENTRACIÓN (L)
Trizma Base	2.49 g
Beijerincks salt 4X (1 lH_2O, 16g NH_4Cl, 2 g $CaCl_2$ x 2H_2O, 4 g $MgSO_4$ x 7H_2O)	25 mL
1M (K)PO_4 pH 7.0	1 mL
Solución de oligoelementos*	1 mL
Ácido Acético	1 mL
H_2O	975 mL

Tabla IV. Composición detallada del medio TAP liquido. **(*** Composición detallada de la solución de oligoelementos en Tabla V).

Las células de *Chlamydomonas* se cultivaban en medio mínimo liquido HS 2, cuya composición por litro esta detallada en la Tabla V. El medio se preparaba a partir de cuatro soluciones concentradas para alcanzar las concentraciones finales indicadas en la Tabla V. Se esterilizaban en autoclave a 121ºC y 1 atm de presión durante 20 min.

Las plantas de *Arabidopsis thaliana* se crecían en macetas con un substrato sólido compuesto por turba y perlita en una proporción 4:1.

1.2.2 Condiciones de cultivo.

Se iniciaban cultivos de *Chlamydomonas* de 50 mL en medio mínimo HS 2 a partir de células creciendo en medio sólido, preparado con medio TAP suplementado con 1.5% de agar. A partir de ese cultivo madre se re-escalaban cultivos de 1 litro que se crecían en las siguientes condiciones:

- alto CO_2: aire mezclado con 5% (v/v) CO_2 y agitación.
- bajo CO_2: aire conteniendo 0.032% (v/v) CO_2 y agitación.

En ambos casos el crecimiento de las células se llevaba a cabo en una cámara de cultivo con un fotoperiodo de 12/12 h luz/oscuridad y una temperatura de 24 º C.

Las plantas de *Arabidopsis* se crecían en condiciones controladas en una cámara de cultivo con un fotoperiodo de 8/16 h luz/oscuridad (día corto) y una temperatura de 24 º durante el día y 18 º C durante la noche.

COMPOSICIÓN	CONCENTRACIÓN (g/L)
HSS	
NH_4	0.5
$MgSo_4 \cdot 7\ H_2O$	0.02
$CaCl_2 \cdot 6\ H_2O$	0.015
$CaCl_2 \cdot 2\ H_2O$	0.010
HSP	
K_2HPO_4	1.44
KH_2PO_4	0.72
Solución Fe-Citrato	
NH_4Fe-citrato	0.05
K_3-citrato	0.08
Solución de oligoelementos	
EDTA	0.05
$ZnSO_4 \cdot 7\ H_2O$	0.02
H_3BO_3	0.01
$MnCl_2 \cdot 4\ H_2O$	$5.1 \cdot 10^{-3}$
$Fe\ SO_4 \cdot 7\ H_2O$	$5.0 \cdot 10^{-3}$
$CoCl_2 \cdot 6\ H_2O$	$1.6 \cdot 10^{-3}$
$CuSO_4 \cdot 5\ H_2O$	$1.5 \cdot 10^{-3}$
$(NH_4)\ Mo_7O_{24}$	$1.1 \cdot 10^{-3}$

Tabla V. Composición detallada del medio HS2.

2. Métodos.

2.1 Técnicas analíticas.

2.1.1 Determinación del contenido en clorofilas.

La medida del contenido en clorofilas se realizaba según el método descrito por Porra y col., (1989), espectrofotométricamente midiendo a dos longitudes de onda, 650 nm (clorofila *b*) y 665 nm (clorofila *a*). Una alícuota de los extractos totales o de cloroplastos aislados se resuspendía en metanol. Las muestras se centrifugaban inmediatamente a 10.000 rpm durante 5 min y el sobrenadante, conteniendo los pigmentos extraídos, se utilizaba para llevar a cabo la determinación de clorofilas.

La concentración de clorofilas totales en mg/mL se determinaba según la fórmula:

$[\text{Clorofila}]_T = 22{,}1 \cdot \text{Absorbancia 650 nm (Clorofila } b\text{)} + 2{,}71 \cdot \text{Absorbancia 665 nm (Clorofila } a\text{)}$

2.1.2 Determinación del contenido en proteína.

La concentración de proteínas en los diferentes extractos celulares se determinaba según el método de Bradford (1976). Una muestra de 800 µL se mezclaba con 200 µL del reactivo de Bradford (Biorad) concentrado. Tras agitar las muestras, se incubaban durante 10 min en oscuridad. A continuación, se determinaba la D.O a 595 nm. La recta patrón se realizaba con distintas concentraciones conocidas de albúmina de suero bovina (BSA) (Merck).

2.1.3 Análisis de la estructura de N-glicano de glicoproteínas.

- Preparación del material: Se partía de 10 litros de cultivo de *Chlamydomonas reinhardtii* de las diferentes cepas utilizadas crecidas en medio liquido HS 2 en condiciones de alto CO_2 y las células se recogían por centrifugación (3900 rpm, 5 min) en la centrifuga Sorvall Rc 5CPlus. El pellet obtenido se resuspendía en 20 mL de tampón fosfato 20 mM, pH: 7.4 y se añadía 1 mL de 25x PIC (cóctel de inhibidores de proteasas (Roche)). A continuación, se rompían las células empleando una prensa French (SLM Amincon instrument) a 1300 Pa y el material obtenido se centrifugaba a 1200 rpm durante 3 min para eliminar restos celulares y a continuación 13000 rpm durante 30 min a 4 ºC en la centrifuga Sorvall Rc 5CPlus. El sobrenadante obtenido se centrifugaba a 45000 rpm durante 1h a 4 ºC en una ultracentrífuga (Centrikon T-2070) para eliminar los restos microsomales. Finalmente, para concentrar las muestras se emplearon Amicones ULTRACEL® 10K (Millipore) centrifugando a 7000 rpm, durante 10 min a 4ºC hasta obtener un volumen final de proteínas solubles de 1 mL.

- Obtención de N-glicanos: los N-glicanos se liberaban de las proteínas por sucesivos tratamientos con Pepsina, PNGasa F y PNGasa A (Bakker y col., 2001b) y se purificaban mediante sucesivas eluciones con agua en una columna de resina C18 Bond Elut (Varian, Palo Alto, CA, EE.UU.) y una columna Carbograf (Altech).

- Acoplamiento de los N-glicanos a 2-Aminobenzamida (2AB): Los N-glicanos purificados fueron acoplados a 2-AB usando el protocolo descrito por Royle y col., (2006). En resumen, los N-glicanos se disolvían en 10 µL de 0.35 M 2-AB en dimetil sulfóxido (DMSO)- ácido acético glacial (7:3 v/v) y 1 M de sodio cianoborohidruro.

- Análisis mediante *Matrix-Assisted Laser Desorption Ionisation-Time Of Flight mass spectra* (MALDI- TOF MS): el análisis de los N-glicanos acoplados a 2-AB se realizaba en un espectrómetro de marca Voyager DE-Pro MALDI-TOF (Applied Biosystems, EE.UU.) equipado con un láser de nitrógeno 337 nm. Se utilizaba una matriz de 2, 5-ácido dihidroxibenzoico. La matriz se mezclaba con

los oligosacáridos disueltos en agua en una relación 1:1 (v / v). Se usaron como controles de calibración mezclas de péptidos y proteínas comerciales (SequazymeTM Peptide Mass Standards *kit*, Applied Biosystems). Los disparos de láser se acumulaban para cada espectro, con el fin de obtener una relación señal / ruido aceptable. La adquisición de los espectros se realizaba con la versión 4 del software ExplorerTM Voyager.

2.2 Técnicas de fraccionamiento celular.

2.2.1 Aislamiento de extractos celulares totales.

Para la obtención de los extractos celulares totales se utilizaba el tampón de extracción de proteínas de Agrisera. Se partía de 1.5 mL de cultivo de *Chlamydomonas* y se recogían las células por centrifugación a 13.400 rpm, 5 min. Las células se sometían a dos fases de congelación/descongelación en nitrógeno líquido y a continuación se añadían 200 µL de Tampón de extracción de proteínas (PEB 1x) compuesto por 400 µL PEB 4x (40% v/v glicerol, Tris-HCl pH 8.5, LDS, EDTA), 64 µL de cóctel de inhibidores de proteasas (PIC) 25x y 1136 µL agua destilada. Las muestras se centrifugaban a 13.400 rpm, durante 3 min a 4º C para eliminar el material insoluble y las células intactas. Por último, se recuperaba el sobrenadante, aproximadamente 200 µL, y se le añadían 10 µL de Ditiotritol (DTT) 1M y se incubaba a 70 º C durante 5 minutos.

En paralelo se procesaba otra muestra que nos permitía estimar la concentración de clorofilas.

2.2.2 Aislamiento de cloroplastos.

Para la obtención de cloroplastos (Villarejo y col., 2001) se partía de cultivos de células de *Chlamydomonas reinhardtii* crecidas en diferentes condiciones. Las células se recogían por centrifugación a 5000 rpm durante 5 minutos a Tº ambiente y se resuspendían en 24 mL de Tampón A (5 mM Hepes-KOH, pH 6.8, 7% (p/v) Polietilenglicol 8000 (PEG), 5 mM $MgCl_2$, 0.5% (p/v) Albumina de suero bovina (BSA)). A continuación, se determinaba la concentración de clorofilas antes de tratar las células con Digitonina (detergente que rompe la membrana celular) en Tampón A, en una relación clorofila/digitonina 4:1 para células crecidas en alto CO_2 y 3:1 para células crecidas en bajo CO_2. Después de la adición de Digitonina las muestras se incubaban en un baño de agua a 30 º C durante 1 min. 06 s. para las células de alto CO_2 y 46 s. para las células de bajo CO_2. Transcurrido el tiempo de incubación, se tomaba una muestra de extracto total que se disolvía en Tampón de carga 2x (Glicerol 80 %, 10 % Dodecilsulfato sódico (SDS), 0.5 M Tris-HCl, β-Mercaptoetanol, Azul de Bromofenol 5 %) y en el resto de la muestra se paraba la incubación con la adición de 30 mL de Tampón A frío, seguida de una suave agitación en un baño de hielo. A partir de este momento, todos los pasos se realizaban a 4º C.

Los extractos celulares se centrifugaban a 3200 rpm durante 5 min, desechando el sobrenadante en el que se encontraban suspendidas mitocondrias y componentes citosólicos. El sedimento obtenido, que contenía los cloroplastos puros, se resuspendía en 40 mL de Tampón B (25 mM KCl, 5 mM $MgCl_2$, 150 mM Manitol, 15 mM Hepes-KOH, pH 7.6, 0. 2 mM K_2HPO_4) + 2 mM K_2-EDTA, produciéndose la unión de las mitocondrias no eliminadas y cloroplastos (*clumpping*). La extractos se centrifugaban a 3200 rpm durante 5 min y el sedimento obtenido se resuspendía en 40 mL de Tampón B + 3 mM $MgCl_2$, produciéndose la separación de cloroplastos y mitocondrias contaminantes (*anticlumping*). Finalmente se centrifugaba la suspensión 5 min a 3200 rpm, eliminando el sobrenadante que contenía mitocondrias y resuspendiendo el sedimento que contenía los cloroplastos en 8 mL de Tampón B + 3 mM $MgCl_2$. La suspensión se depositaba sobre un gradiente de Percoll de dos fases: una fase de 4 mL de Percoll 75% (v/v) [7.5 mL Percoll 100 % (v/v), 2 mL Tampón B 5x (125 mM KCl, 5 mM $MgCl_2$, 750 mM Manitol, 75 mM Hepes-KOH, pH 7.6, 1 mM K_2HPO_4) y 1 mL ddH_2O] y otra fase de 4 mL de Percoll 45% (v/v) (4.5 mL Percoll 100 % (v/v) , 2 mL Tampón B 5x y 3.5 mL ddH_2O). El gradiente se centrifugaba a 7500 rpm sin freno y con aceleración lenta, durante 20 min, obteniendo diferentes bandas, de las cuales únicamente se seleccionaba la interfase entre el Percoll al 75 % y al 45 % donde se encontraban los cloroplastos y tilacoides. La banda seleccionada se diluía en 50 mL de tampón de lavado 15 mM Hepes-KOH pH 7.2 y se centrifugaba a 3200 rpm durante 3 min., obteniendo un sedimento que se resuspendía de nuevo en tampón de lavado.

A continuación, se realizaba un segundo gradiente de Percoll procediendo de la misma forma descrita hasta la obtención de la fracción final de cloroplastos purificados.

2.2.3 Obtención de fracciones estromáticas a partir de cloroplastos aislados.

Los cloroplastos aislados se rompían mediante pulsos (30s) en un sonicador (Branson Sonifier 450) y tras una centrifugación a 13.400 rpm durante 20 min a 4 ºC, se obtenía la fracción estromática.

2.2.4 Obtención de fracciones periplásmicas.

Para la obtención de fracciones periplásmicas se partía de 3L de cultivos de células de *Chlamydomonas reinhardtii* crecidas en condiciones de alto CO_2. Las células se recogían por centrifugación a 3900 rpm durante 5 minutos a 4 ºC y se resuspendían en 50 mL de Tampón fosfato 20mM pH 7.4. El sedimento obtenido se resuspendía en 25 mL de Tampón fosfato + 0.4M KCl + 1mL PIC 25X e incubábamos con agitación durante 10 mina 4 ºC. Transcurrido el tiempo de incubación y tras una centrifugación a 3900 rpm durante 5 min a 4 ªC, se obtenía la fracción periplásmica.

2.2.5 Obtención de protoplastos de células de mesófilo de *Arabidopsis thaliana.*

Para la obtención de protoplastos de *Arabidopsis,* las plantas se crecían en las condiciones descritas anteriormente durante 5-6 semanas. Se seleccionaban 10 plantas de *Arabidopsis* (de cada línea) y sus hojas se cortaban en tiras de un tamaño aproximado de 1 mm. Las muestras se digerían en 60 mL de solución de enzima, previamente preparada, con el fin de digerir la pared celular y obtener la cantidad de protoplastos adecuados.

Para la preparación de la solución de enzima se mezclaban 0.5 g celulasa R10 (1 % (p/v)), 0.1g macerozima R10 (0.2 % (p/v)), 25 mL 0.8 M Manitol (0.4 M), 2 mL 0.5 M KCl (20 mM), 2 mL 0.5 M MES pH 5.7 (20 mM)), se calentaba 55ºC durante 10 min antes de añadir 0.5 mL $CaCl_2$ (10 mM), 0.05 g BSA (0.1 % (p/v)) y 20.5 mL de agua destilada. Por último, la solución de enzima se filtraba a través de un filtro con diámetro de poro de 0.45 μm.

Una vez añadidas las hojas en tiras de 1 mm en la solución de enzimas, se aplicaba vacío a las muestras durante 15 min y se continuaba la digestión durante 1 h agitando las muestras a 40 rpm. Transcurrido este tiempo, la solución de enzima conteniendo los protoplastos adquiría una tonalidad verde, como consecuencia de la liberación de los protoplastos y se filtraba a través de una malla de nylon con diámetro de poro de 41 μm. El filtrado se centrifugaba a 900 rpm durante 2 min y el sedimento, que contenía los protoplastos intactos, se lavaba con 5 mL de solución W5 (154 mM NaCl, 125mM $CaCl_2$, 5 mM KCl, 2 mM MES pH 5.7). Los protoplastos se centrifugaban a 700 rpm durante 2 min y se realizaba otro lavado con la solución W5. A continuación, se incubaba en hielo durante 30 min. Transcurrido el tiempo de incubación, se centrifugaban las muestras a 700 rpm durante 2 min y se resuspendían en 5 mL de solución MMg (0.4 M Manitol, 15 mM $MgCl_2$, 4 mM 0.5M MES pH 5.7). Para finalizar, se contaban los protoplastos en una cámara Neubauer para estimar el número de protoplastos por mL y obtener una concentración adecuada para realizar la transfección. La concentración final óptima de los protoplastos para realizar la transfección era $4 \cdot 10^5$ células/mL.

2.3 Técnicas bioquímicas.

2.3.1 Determinación de actividades enzimáticas.

Para detectar la posible contaminación de las preparaciones de cloroplastos con peroxisomas y mitocondrias, se realizaban medidas de actividades enzimáticas marcadoras de dichos compartimentos celulares. Como marcador de mitocondria se utilizaba la enzima Fumarasa (EC 4.2.1.2), que cataliza la conversión de Malato a Fumarato en el ciclo de Krebs. Como marcador de peroxisomas se utilizaba la enzima

Hidroxipiruvato reductasa (EC 1.1.1.81), que cataliza la conversión de Hidroxipiruvato a Piruvato.

La medida de actividad Fumarasa se llevaba a cabo en una mezcla de reacción (1 mL, volumen final) que contenía 50 mM tampón Tricina-NaOH (pH 7,5) y extracto celular total o cloroplastos aislados (7 μg de clorofila). La reacción se comenzaba con la adición de 50 mM Malato. La actividad fumarasa se determinaba espectrofotométricamente a 240 nm y 22ºC (Hill y Bradshaw, 1969).

La medida de hidroxipiruvato reductasa (HPR) se realizaba en una mezcla de reacción (1 mL, volumen final) que contenía 50 mM tampón MES-NaOH (pH 6.4), 200 μM NADH, y extracto celular total o cloroplastos aislados (7 μg de clorofila). La reacción se comenzaba con la adición de 1 mM malato. La actividad HPR se ensayaba espectrofotométricamente a 340 nm y 22ºC (Tolbert y col., 1970). La medida del cambio de absorbancia a esta longitud de onda permite hacer un seguimiento de la oxidación del NADH a NAD^+, que es proporcional a la actividad de la HPR.

2.3.2 Tratamiento con inhibidores.

Se partía en todos los casos de 1 litro de cultivo de *Chlamydomonas* y se trataban con 3 inhibidores diferentes que bloquean el tráfico de las proteínas en diferentes puntos:

- Monensina: su efecto es inhibir la salida de vesículas desde el Golgi, bloqueando el tráfico vesicular (Boss y col., 1984). Se preparaba un stock de 10 mM en etanol, se añadía a una concentración final de 1 μM y los cultivos se incubaban durante 2 horas con agitación.
- Brefeldina A (BFA): inhibe el transporte entre el RE y el sistema de Golgi (Gómez y Chrispeels, 1993). Causa un bloqueo en el sistema secretor de las células eucariotas por inhibir el transporte retrógrado de vesículas al aparato de Golgi (Ritzenthaler y col., 2002). Los cultivos se incubaban con 25 μM de BFA durante 24 h con agitación. El inhibidor se añadía a partir de un stock de BFA 36 mM disuelto en DMSO.
- Tunicamicina: Es un inhibidor de la glicosilación de las proteínas en el RE (Misoon y col., 2004). Se preparaba en etanol a una concentración de 10 mg/mL, se añadía a una concentración final de 10 μg/mL y los cultivos se incubaban durante 24 horas con agitación.

Posteriormente se aislaban extractos celulares totales y cloroplastos de las células tratadas con los diferentes inhibidores.

2.3.3 Digestión con Endoglicosidasa H y N-peptidilendoglicosidasa F y A.

Las N-glicoproteínas presentes en los extractos celulares totales, los de cloroplastos y las fracciones estromáticas fueron digeridas con Endoglicosidasa- H (Endo H) y N-peptidilendoglicosidasa F (PNGasa F) y N-peptidilendoglicosidasa A (PNGasa A):

Endo H es una enzima aislada de una cepa de *E.coli* recombinante. Su actividad consiste en hidrolizar el enlace entre las dos moléculas de GlcNAc proximales del *core* de los N-glicanos de glicoproteínas (Figura VIII). La enzima Endo H es activa sobre oligosacáridos de alto contenido en manosa.

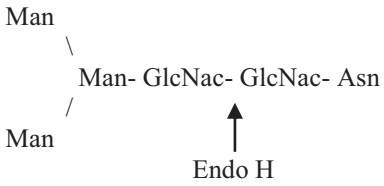


Figura VIII. Digestión con Endo-H.

PNGasa F es también una enzima recombinante comercial que hidroliza el enlace N-glicosídico entre la GlcNAc proximal del "core" de quitobiosa del N-glicano y el residuo de asparragina (Asn) de la cadena polipeptídica (Figura IX). Es activa sobre todo tipo de cadenas de N-glicanos unidos a péptidos o proteínas, salvo que estén bloqueadas con residuos de fucosa en enlace α(1,3), lo cual supone un impedimento estérico.

PNGasa A hidroliza el enlace N-glicosídico entre las GlcNAc y el residuo de Asn de la cadena polipeptídica (Figura IX) y a diferencia de la enzima PNgasa F, es activa sobre todo tipo de cadenas unidas N-glicosidicamente a glipéptidos aunque presenten residuos de fucosa en el enlace α(1,3).

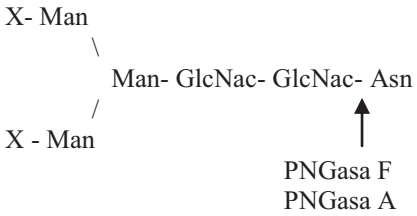


Figura IX. Digestión con PNGasa F y PNGasa A.

Las muestras se digirieron siguiendo las indicaciones del fabricante. Las condiciones de la reacción de digestión y el procedimiento a seguir se indican en el Tabla VI.

Una vez transcurrido el tiempo de digestión, se añadía a las muestran tampón de carga 2x y se calentaban a 95º C durante 10 min. Posteriormente las muestras se analizaban utilizando diversas técnicas de identificación como se verá en el punto 2.5 (Western Blot y Afinoblot).

PNGasa F	**Endo H**
1-20 µg glicoproteínas	1-20 µg glicoproteínas
1 µL Tampón de desnaturalización de glicoproteínas 10x	1 µL Tampón de desnaturalización de glicoproteínas 10x
X µL H_2O	X µL H_2O
Volumen: 10 µL	Volumen: 10 µL
⬇	⬇
INCUBACIÓN 10 MIN, 100ºC	INCUBACIÓN 10 MIN, 100ºC
⬇	⬇
2 µL Tampón reacción G7 10x	2 µL Tampón reacción G5 10x
2 µL 10% Nonidet- P40 (NP40)	-
1 µL PIC 25x	1 µL PIC 25x
X µL H_2O	X µL H_2O
1 µL Endo H	1 µL PNGasa
Volumen final: 20 µL	Volumen final: 20 µL
⬇	⬇
INCUBACIÓN 24 h, 37ºC	INCUBACIÓN 24 h,37ºC

Tabla VI. Reacción de digestión con Endo H y PNGasa F.

2.4 Técnicas de genética molecular.

2.4.1 Aislamiento de ADN genómico.

El ADN se extraía de células de *Chlamydomonas* creciendo en fase exponencial. Las células procedentes de 30 mL de cultivo se recogían mediante centrifugación a 5000 rpm durante 5 min a Tª ambiente. El sedimento se resuspendía en 15 mM Hepes pH 7.0 y tras una nueva centrifugación, el sedimento obtenido se resuspendía en 3 mL de agua destilada estéril más 4 mL de Tampón CTAB (*Cetyl trimethylammonium bromide*) (Sigma), (2% (p/v) CTAB 100 mM Tris-HCl, pH 8; 20 mM EDTA; 1.4 M NaCl; 2% (v/v) β-mercaptoetanol). La suspensión se incubaba durante 30 min a 60 ºC y se esperaba a que descendiera la temperatura. Acto seguido se añadían 7 mL de cloroformo, agitando durante 5 min. Tras una centrifugación a 5000 rpm durante 5 min

a Tª ambiente, se eliminaba el sobrenadante y se repetía el tratamiento con cloroformo. El sedimento obtenido se resuspendía con 7 mL de isopropanol y se incubaba en hielo durante 30 min. Transcurrido el tiempo de incubación, se centrifugaba a 10.000 rpm durante 6 min y se lavaba el pellet con 2 mL de etanol 80%. Se repetía la centrifugación a 10.000 rpm, eliminando por completo el etanol y se añadían 200 µL de Tampón TE (Tris 10 mM; EDTA 1 mM pH 8) y 5 µL de RNAsa (10 mg/mL) (Sigma). Las muestras se incubaban durante 30 min a 37 ºC. A continuación, se añadían 1/10 volumen de acetato potásico y 2 volúmenes de etanol 100 % (v/v) con el fin de precipitar el ADN. Se incubaban en hielo durante 5 min, se centrifugaban y se eliminaba todo el sobrenadante, lavando el sedimento obtenido con etanol 80 % (v/v). Por último se eliminaba todo el etanol por centrifugación y se secaban los tubos a temperatura ambiente, resuspendiendo el sedimento de ADN en 50 µL de agua destilada estéril.

2.4.2 Aislamiento de ADN plasmídico.

El ADN plasmídico se aislaba a partir de 3 mL de cultivo procedentes de colonias de *E.coli* crecidas en LB líquido (5 g/L de Extracto de levadura, 10 g/L de Triptona, 5 g/L de Cloruro sódico) con el antibiótico adecuado durante toda la noche a 37º C con agitación. Para la extracción del ADN plasmídico se siguieron las instrucciones indicadas en el *kit* de extracción RapidPURE Plasmid Mini kit (Q-bio gene).

2.4.3 Aislamiento de ARN total.

El ARN se extraía de células de *Chlamydomonas* en fase exponencial crecidas en medio HS2 o TAP según fueran las necesidades de cada experimento concreto. Las células procedentes de 30-60 mL de cultivo se recogían mediante centrifugación a 5.000 rpm durante 10 min a temperatura ambiente. El sedimento se lavaba con tampón 15 mM Hepes pH 7.5, se centrifugaba a 5.000 rpm durante 10 min a Tº ambiente y al sedimento obtenido se le añadía 1 mL de Trizol (Invitrogen). La muestra homogeneizada se incubaba a 30 ºC durante 5 min para permitir la completa disociación de los complejos nucleoprotéicos y a continuación se añadían 200 µL de cloroformo para precipitar las proteínas. Se agitaba vigorosamente durante 15 s y se incubaba a 28 ºC durante 3 min. A continuación, se centrifugaba la muestra a 13.400 rpm durante 15 min a 4 ºC. La mezcla se separaba en diferentes fases: una fase de cloroformo inferior, una fase intermedia y una fase acuosa superior, permaneciendo el ARN exclusivamente en la fase acuosa.

Se transfería la fase acuosa a un tubo nuevo y se mezclaba con 500 µL de isopropanol. La muestra se incubaba a 28 ºC durante 10 min y el sedimento de ARN obtenido tras centrifugación a 13.400 rpm durante 15 min a 4 ºC se lavaba con 1 mL de etanol 75 %. Se agitaba la muestra y se centrifugaba a 13.400 rpm durante 15 min a 4ºC. Se eliminaba el sobrenadante y se secaba el sedimento de ARN durante aproximadamente 15 min a temperatura ambiente. Para finalizar se disolvía el ARN en

50 µL de agua destilada estéril libre de RNAsas tras ser tratada con Dietilpirocarbonato (DEPC (Merck)).

2.4.4 Síntesis de ADNc.

Para la síntesis de ADNc se siguieron las instrucciones del *kit* First Strand cDNA Synthesis (Fermentas). Se preparaba la siguiente mezcla de reacción: 1 µL de ARN (0.1-5 µg), 1 µL cebador oligo(dT) (0.5 µg/ µL) y 9 µL de agua tratada con DEPC. Se agitaba y se incubaba la mezcla a 70 ºC durante 5 min. Se ponía en hielo y se añadía 4 µL de tampón de reacción 5x, 1 µL Ribolock Ribonuclease Inhibitor (20U/ µL) y 2 µL mezcla de dNTP10 mM, a continuación, se incubaba a 37 ºC durante 5 min. Para finalizar se añadía 2 µL de M-MuLV transcriptasa inversa (20u/ µL), se incubaba la mezcla a 37 ºC durante 60 min y se paraba la reacción a 70 ºC durante 10 min. El ADNc se podía usar directamente para la amplificación por PCR.

2.4.5 Amplificación de ADN por PCR.

Se amplificaba el ADN mediante la técnica de PCR (Reacción en cadena de la polimerasa). Todos los cebadores utilizados y su temperatura de anillamiento (Tm) están detallados en la Tabla VII.

NOMBRE	SECUENCIA 5´- 3´	T.ANILLAMIENTO (ºC)
FT-exón 5´ F	ACTGGCTCGTTCGACAAGAT	56.5
FT-exón 7´ R	CACTTGCGCGACTTCCAC	58.5
FT-RACE 5´	CTCCATCTTGTCGAACGAGCCAGT	64.0
FT-RACE 3´	CTGTTCGACCCCGAGTGGAC	61.6
FT-tagF	GCGCAT**CCCGGG**AACGTTAAGAAGCACCACCC	79.1
FT-tagR	CGTA**AGATCT**CTTGACGTGGTAGTCCTTGG	65.4
α-ManIF-2	GGCATTACCCCTACCAGGAT	56.5
α-ManIR-2	GCGTACTCCTCCAGCAGACC	59.0
FT-ClonF	AACGTTAAGAAGCACCACCCC	59.9
FT-ClonR	TGCCATTCGCCCTTTACA	56.8
B2TUBF	GAGTTCACTGAGGCCGAGTC	63.0
B2TUBR	ATCCGACGAGATGAATGTCC	58.0
RT-FT F	TGCACTTACAACACGCATCA	56.0
RT-FT R	TTGGGGTGGTGCTTTTTAAC	58.0
ManII F	TGGGTGGAGATGGACACGAA	59.9
ManII R	CAGTAGAAGGTGGTGTGCGG	58.8
XylT F	AACACCCCAACACCAATCGG	61.8
XylT R	CAGCTCCTTGTGGGGGTAGA	59.1
FT8 F	GCTTCATCTACCGCCACCAG	59.3
FT8 R	CGAGAAGGGCAGGAAGATGC	60.3
FT8-RACE 5´	GATGGCAGGCAGCTGTACAACAG	61.3
FT8-RACE 3´	AACCTGTTCTACGACTCACGGTAC	61.8

Tabla VII. Oligonucleótidos cebadores empleados para las diferentes reacciones de PCR y RT-PCR. Se resaltan en el oligonucleótido FT-tagF y FT-tagR sitios de restricción adicionales para las enzimas *SmaI* (CCCGGG) *y BglII* (AGATCT).

Todas las reacciones se realizaban partiendo del ADN aislado y usando los reactivos de Biotools. Los volúmenes utilizados se muestran en la Tabla VIII.

Reactivos	dNTPs (10mM)	Tampón 10x	$MgCl_2$ (50mM)	Cebadores (10µM)		DNA polimerasa (1U/ µL)	H_2O	ADN	**Volumen final**
				Directo	Reverso				
Volumen (µL)	0.5	2.5	1	1	1	1	x	x	25

Tabla VIII: Reactivos utilizados para la reacción de PCR.

El programa de PCR utilizado para la amplificación de los fragmentos de ADN se muestra en la Tabla IX.

También se realizaron reacciones de PCR con gradientes de temperatura utilizando un termociclador de Bio-Rad modelo MJ Mini Personal Thermal Cycler.

Fases	Desnaturalización		Anillamiento	Elongación		Mantenimiento
Temperatura (ºC)	94	94	Tm*	72	72	14
Tiempo	5 (min)	30 (s)	30 (s)	30(s)-1(min)	7 (min)	Indefinido

30-35 ciclos

Tabla IX: Programa del termociclador para la amplificación de los fragmentos de ADN.

*: Temperatura de anillamiento menos 2 ºC con respecto a la Tm específica de cada par de cebadores.

También se realizaron reacciones de PCR con el *kit* FastStart High Fidelity PCR System (Roche). Este sistema dispone de una polimerasa más eficiente en la ratio de errores cometidos al replicar el molde que la polimerasa suministrada por Biotools y permite la amplificación de una variedad de fragmento de ADN y ADNc de hasta 5 kb y aumentar la fidelidad. Igualmente, se siguieron las instrucciones del fabricante.

Todas las reacciones se realizaban partiendo del ADN aislado y usando los reactivos del *kit*. Los volúmenes utilizados se muestran en la Tabla X.

Volúmen (µL)	**Componentes**
5 µL	H_2O
5 µL	Tampón de reacción FastStart High Fidelity 10x
1 µL	DMSO
1 µL	Mezcla de dNTP (10 mM)
1 µL	Cebador Directo (10 µM)
1 µL	Cebador Reverso (10 µM)
1 µL	ADN
0.5 µL	Mezcla FastStart High Fidelity Enzyme
Volumen final: 50 µL	

Tabla X. Reactivos utilizados para la amplificación del ADN con el kit FastStart High Fidelity.

El programa de PCR utilizado para la amplificación de los fragmentos de ADN con el *kit* FastStrat High Fidelity se muestra en la Tabla XI.

Fases	Desnaturalización		Anillamiento	Elongación		Mantenimiento
Temperatura (ºC)	95	95	Tm*	72	72	14
Tiempo	2 (min)	30 (s)	30 (s)	30(s)-1(min)	7 (min)	Indefinido
		35 ciclos				

Tabla XI. Programa del termociclador para la amplificación de ADN con el kit FastStart High Fidelity PCR.

2.4.6 RT-PCR

La RT-PCR (*Retro Transcriptase Polimerase Chain Reaction*) es una técnica delicada y versátil que se utiliza para medir la expresión génica de tejidos y cultivos celulares (Powell y col., 1987; Wilkinson, 1998). Tradicionalmente, la RT-PCR se realiza en dos reacciones. En la reacción inicial, se sintetiza ADNc a partir de ARN y en una reacción separada, el ADNc se amplifica por PCR utilizando una ADN polimerasa termoestable. Estos dos pasos requieren múltiples medidas de manipulación, entre ellas la dilución seriada del ADNc en múltiples tubos y la adición secuencial de enzimas y otros reactivos a cada tubo. En cambio, el uso del *kit* TITANIUM™One-Step RT-PCR (Clontech) permite la síntesis de ADNc y la realización de la PCR en un único tampón con una única mezcla de enzimas. Este método reduce la posibilidad de contaminación cruzada y ofrece una técnica conveniente para estudios de expresión génica.

La RT-PCR se llevaba a cabo siguiendo las instrucciones del *kit* TITANIUM™One-Step RT-PCR (Clontech) y con oligonucleótidos cebadores específicos (Tabla VII).

La mezcla de reacción se indica en la Tabla XII y el programa utilizado para la realización de la RT-PCR se indica en la Tabla XIII.

Volumen (µL)	**Compuesto**
1-5.5 µL	ARN (1ng-1µg)
1 µL	Cebador Directo (45 µM)
1 µL	Cebador Reverso (45 µM)
43.5 µL	Mezcla de reacción
X	H_20 libre de RNAsa
Volumen final: 50 µL	

Mezcla de Reacción	
Volumen (µL)	**Compuesto**
5 µL	Tampón "One-Step"10x
1 µL	Mezcla dNTP50x
0.5 µL	"Recombinant RNAsa Inhibitor"
25 µL	"Thermostabilizing Reagent"
10 µL	"GC-Melt™"
1 µL	Cebador Oligo(dT)
1 µL	Mezcla RT-TITANIUM™ Taq Enzyme 50x
Volumen Total: 43.5 µL	

Tabla XII. Reactivos utilizados para la RT-PCR.

Fases	Retrotranscripción	Desnaturalización		Anillamiento	Elongación		Mantenimiento
Temperatura (ºC)	50	94	94	65	68	68	14
Tiempo	1(h)	5 (min)	30 (s)	30 (s)	1-1.5(min)	2 (min)	Indefinido

30-35 ciclos

Tabla XIII. Programa del termociclador para la RT-PCR.

2.4.7 Técnica de Amplificación rápida de extremos de ADNc (RACE).

Para la obtención de la secuencia completa de aquellos genes de los que sólo se conocía una parte del mismo, se utilizaron técnicas de RACE (Rapid Amplification of cDNA Ends). Con este fin se usó el *kit* BD SMART™ RACE cDNA Amplification (BD Biosciences Clontech) siguiendo las instrucciones del fabricante.

El primer paso era la síntesis de ADNc-5´y ADNc-3´ a partir del ARN aislado de cultivos en crecimiento exponencial de *Chlamydomonas*, creciendo en distintas condiciones. El procedimiento seguido se indica en el Tabla XIV.

ADNc-5´	**ADNc-3´**
1 µL ARN (1 µg/ µL)	1 µL ARN (1 µg/ µL)
1 µL Cebador 5´-CDS	1 µL Cebador 3´-CDS
1 µL Oligo 5´-BD SMART II A	--------
2 µL H_2O	2 µL H_2O

↓

INCUBACIÓN 70ºC, 2 MIN
INCUBACIÓN HIELO, 2MIN

↓

Se añadía en cada reacción:

2 µL TAMPÓN FIRST-STRAND 5X
1 µL DTT (20 mM)
1 µL Mezcla dNTP (10mM)
1 µL Transcriptasa inversa BD PowerScript
Volumen total: 10 µL

↓

Incubación 42ºc, 1.5 h
Se diluyó el producto de reacción con 100 µL de tricina-edta
Incubación 72ºc, 7 min

Tabla XIV. Síntesis de ADNc-5´y ADNc-3´con kit BD SMART™RACE cDNA Amplification. *Cebador 5´-CDS y cebador 3´-CDS suministrados en el kit.*

Una vez obtenidos los ADNc se procedía a la realización de las reacciones de PCR como se indica en la Tabla XV. El programa de PCR utilizado para la amplificación de los ADNc con BD SMART™ RACE cDNA Amplification se muestra en la Tabla XVI.

Volumen (µL)	**RACE-5´**	**RACE-3´**
2.5 µL	ADNc-5´	ADNc-3´
5 µL	UPM (10x)	UPM (10x)
1 µL	Cebador Específico (10µM)	Cebador Específico (10µM)
41.5 µL	Mecla de Reacción	Mezcla de reacción
Volumen final: 50 µL		

→

Mezcla de Reacción	
Volumen (µL)	**Compuesto**
34.5 µL	Agua PCR-Grade
5 µL	Tampón BD Advantage 2 PCR 10x
1µL	Mezcla dNTP (10mM)
1 µL	50x BD Advantage 2 Polymerase Mix
Volumen Total: 41.5 µL	

Tabla XV. Reactivos utilizados para la amplificación de los ADNc-5´ y ADNc-3´ con kit BD SMART™RACE cDNA Amplification. UPM: cebador universal A (10x): 5´-CTAATACGACTCACTATAGGGC-3´, suministrados en el *kit.*

Fases	Desnaturalización		Anillamiento	Elongación		Mantenimiento
Temperatura (ºC)	94	94	68	72	72	14
Tiempo	5 (min)	30 (s)	30 (s)	3 (min)	7 (min)	Indefinido

25 ciclos

Tabla XVI. Programa del termociclador para la amplificación de los ADNc-5´ y ADNc-3´.

2.4.8 Aislamiento y purificación de fragmentos de ADN.

Los fragmentos ADN seleccionados eran recortados y extraídos de los geles de agarosa y se purificaban utilizando el *kit* QIAEX®II Gel Extraction kit (QIAGEN).

2.4.9 Tratamiento enzimático del ADN.

Las digestiones del ADN (tanto genómico como plasmídico) mediante enzimas de restricción se realizaban de acuerdo con las instrucciones del fabricante (Takara), en cuanto a los tampones utilizados como a los tiempos y Tª de incubación.

En el caso de digestiones dobles con tampón incompatible, se realizaban de forma secuencial. Una digestión doble con las enzimas de restricción *Bgl II* y *Sma I* (Takara) se muestra en la Tabla XVII.

Compuesto	ADN	Buffer T	*Sma I* (10U/µL)	BSA	Buffer H	*Bgl II* (10U/µL)
Volumen (µL)	20	3	1	3	1.5	1

1h, 30ºC 1h, 37ºC

Tabla XVII. Reacción de doble digestión de DNA.

A partir del ADN plasmídico se realizaban digestiones, tanto para aislar los fragmentos que se usarían en futuros clonajes como para comprobar la presencia del fragmento de interés en el vector, tras los procesos de transformación.

2.4.10 Reacciones de ligación y clonaje.

Los fragmentos a analizar se clonaban en el vector pGEM T-Easy siguiendo las instrucciones del *kit* PGEM®T-easy vector (Promega) Tabla XVIII. La reacción de ligación se incubaba durante 1 h a Tª ambiente.

Compuestos	Buffer ligación 2x	Vector pGEM T-Easy	ADN	T4 DNA ligasa 3U/μL
Volumen (μL)	5 μL	1 μL	3 μL	1 μL

Tabla XVIII. Mezcla de reacción de ligación para fragmentos de DNA.

2.4.11 Transformación de células competentes.

Los vectores plasmídicos con los fragmentos clonados que se describen en cada caso eran introducidos en células competentes de *E.coli* DH5α, preparadas según la modificación descrita por Kushner (1978) del método del cloruro cálcico. Se añadían 10 μL de mezcla de ligación (plásmido con inserto de interés) a una alícuota de 200 μL de células competentes de *E.coli* DH5α. Se incubaba esta mezcla en hielo durante 30 min, para permitir que las moléculas de DNA entraran en contacto con las células competentes. A continuación, se daba un choque de calor por incubación a 42 ºC durante 2 min, seguido de una incubación en hielo durante 1 min (choque de frío). Este tratamiento altera las la membrana externa aumentando su permeabilidad, facilitando de esta forma la incorporación del DNA. Se añadían 800 μL de medio LB a temperatura ambiente y se incubaba durante 1 hora en agitación (250 rpm) a 37 ºC.
Después de este tiempo de incubación, distintas diluciones de las células transformadas se plaqueaban en medio LB sólido con el antibiótico adecuado y se incubaban a 37 ºC durante toda la noche.

2.4.12 Etiquetado con epítopo de hemoaglutinina.

Para llevar a cabo el etiquetado con el epítopo de Hemoaglutinina (HA) del clon de la α(1,3) fucosil-transferasa de *Chlamydomonas*, se utilizó el plásmido pPE 1000 (Figura X) (Hancock y col., 1997).

El plásmido pPE 1000 incorpora el fragmento marcador que codifica para HA y permite la incorporación de la región que codifica para HA, bien en el extremo 5´ o bien en el extremo 3´. Además permite la utilización de dos sitios de restricción (*BglII* y *SmaI*, en nuestro caso) que permiten la orientación del fragmento en el sentido adecuado. En nuestro caso, nos interesaba la incorporación de la región codificante de HA en el extremo 3´.

Se comenzó amplificando la región codificante del gen de la fucosil-transferasa 1 (*crft1*) de *Chlamydomonas* mediante reacciones de PCR usando el *kit* FastStart High Fidelity PCR System y utilizando los cebadores específicos, FT-tag R y FT-tag F descritos anteriormente en la Tabla VII. Estos cebadores permitían incluir sitios de restricción para *BglII* y *SmaI* y modificaban el codón de terminación en el extremo 3´ del gen.

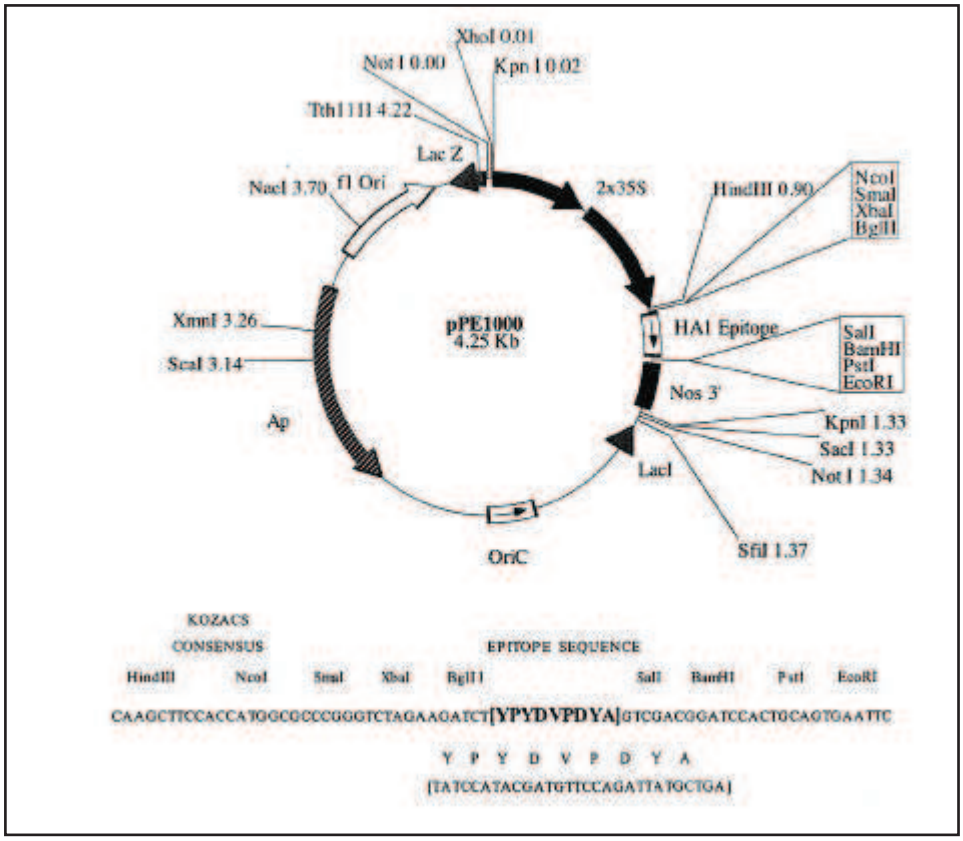


Figura X. Mapa de restricción del plásmido pPE 1000. *Imagen obtenida del artículo de Hancock y col., 1997.*

Una vez obtenido el fragmento, con los sitios de restricción adecuados y el codón de terminación modificado, se realizaban dobles digestiones con las enzimas de restricción *BglII* y *SmaI*, tanto del DNA de fucosiltransferasa 1 como del DNA plasmídico del vector pPE 1000. Una vez digerido y purificado el DNA se procedía a la ligación.

El vector final, al que denominamos pBG 3074 conteniendo el clon de fucosiltransferasa 1 etiquetado con el epítopo de HA, fue seleccionado tras la transformación en *E.coli* DH5α y secuenciación del ADN plasmídico para comprobar la orientación y la fidelidad de lectura.

2.4.13 Transfección de protoplastos con Polietilenglicol (PEG).

Los protoplastos obtenidos a partir de hojas de plantas de *Arabidopsis* (ver apartado 2.2.4) se transfectaron con el ADN de *Chlamydomonas* clonado en el vector pBG 3074.

Se utilizaban 10 µL de ADN (14 µg de ADN plasmídico) y 100 µL de protoplastos ($4\cdot 10^4$ células) de las distintas estirpes de *Arabidopsis*. Se añadían a la muestra 110 µL de PEG 4000 para permitir la entrada del DNA a los protoplastos, se

mezclaba y se incubaba a 23 ºC durante 15 min. Transcurrido el tiempo de incubación, se añadían 440 µL de la solución W5 descrita en apartado 2.2.4, se centrifugaba a 1000 rpm durante 1 min, se eliminaba el PEG y se resuspendían los protoplastos en 500 µL de solución W5. Se incubaban las muestras en placas multipocillo a 24 ºC durante 24 h. Transcurrido este tiempo se procedía a la recogida de las muestras y se guardaban a –20 ºC para su posterior análisis.

2.4.14 Silenciamiento del gen *crft1* de *Chlamydomonas* mediante miARNs artificiales.

Para la obtención de líneas de *Chlamydomonas* con el gen *crft1* silenciado se llevó a cabo la técnica de silenciamiento mediante miARNs artificiales descrito por Molnar y col., 2009; 2007 y Zhao y col., 2009; 2007.

2.4.14.1 Construcción de miARN artificales.

Para la construcción del miARN artificial en *Chlamydomonas* se seleccionó como precursor del miARN artificial el microARN cre-MIR1157 (número de acceso MI0006219) (Molnar y col., 2007; 2009) como esqueleto para expresar el miARN artifical (amiARN) dado que posee horquilla corta (137 nt) y es abundante en células vegetativas.

Se empleó el vector pChlamiRNA2 (Figura XI) cedido por el Prof. Baulcombe (Univ. De Cambridge, UK) que incorpora el precursor miARN cre-MIR1157 bajo el control del promotor constitutivo en tándem *HSP70A-RBCS2* (Schoda y col., 1999). Este promotor *HSP70A* sirve como activador de la transcripción cuando se coloca por encima del promotor del gen *RBCS2* que codifica para la subunidad pequeña de la ribulosa-1,5-bifosfato carboxilasa/oxigenasa (RuBisCo), una enzima clave para la fijación de CO_2 durante la fotosíntesis. Y posee como terminador de la transcripción 3` UTR de *RPL12* (uno de los 3´-UTRs de *Chlamydomonas* más cortos (Merchant y col., 2007) Este vector incluye la secuencia de un terminador invertido del tránscrito estExt_fgenesh2_pg.C_310026 (protein ID 192566) (C310026 3´UTR) para terminar la transcripción de cualquier transcrito antisentido en el sitio de integración del transgen. También posee el gen ARG7 usado como marcador de selección, que permitiría a líneas celulares auxotróficas para arginina crecer en ausencia de la misma y también posee el gen que confiere resistencia a Ampicilina como método de selección en *E.coli*. Este vector contiene además, un sitio de corte para *SpeI* que flanquea miRNA*–spacer–miRNA, permitiendo así el clonaje, siendo miRNA la secuencia seleccionada del gen de interés y miRNA* la secuencia complementaria (mirar figura 2.9, capitulo 2 de resultados) de acuerdo a Molnar y col., 2009.
(http://www.plantsci.cam.ac.uk/Baulcombe/protocols.htmL).

La secuencia de cre-MIR1157 se modificó en este vector para producir un amiARN.

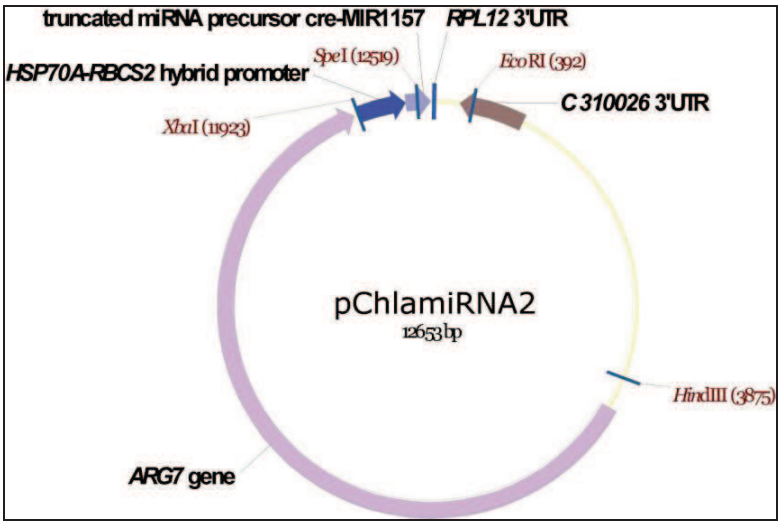


Figura XI. Vector artificial miARN pChlamiRNA2. *Figura tomada de www.http://chlamycollection.org/plasmid/pchlamirna2/.*

Usamos el software WMD3 (web MicroRNA Designer platform) http://wmd3.weigelwordl.org/cgi-bin/cgi?pag=Home;project=stdwmd (Ossowski y col, 2008) para identificar regiones de nuestro gen de interés susceptibles de ser candidatas para el silenciamiento. La construcción del vector de miARN artificial de *Chlamydomonas* para silenciar el gen *crft1* se realizó de acuerdo a las instrucciones detalladas descritas por Molnar y col., 2009. Fueron seleccionados distintos cebadores, aunque solo los indicados a continuación fueron efectivos: (en negrita se indica las secuencias de miRNA y subrayados miRNA*) (Tabla XIX).

FT1miFor_3	ctagt**AAGCTGTGACGTGAGTGTAAA**tctcgctgatcggcaccatgggggtggtggtgatcagcgctaTTTATGCTCACGTCACAGCTTg
FT1miRev_3	ctagc**AAGCTGTGACGTGAGCATAAA**tagcgctgatcaccaccaccccatggtgccgatcagcgagaTTTACACTCACGTCACAGCTTa

Tabla XIX. Secuencia de cebadores empleados para la preparación de miARNs para crft1, que produjo cepas silenciadas.

2.4.14.2. Clonación de los cebadores dsADN para la expresión de miARN artificial.

Los cebadores ssADN se diseñaron para remplazar el ADN de la horquilla de miARN sin la necesidad de reacciones de PCR e incluyen un sitio de restricción para *SpeI*, permitiendo de esta manera la ligación en el sitio *SpeI* del vector pChlamiRNA2.

Los cebadores ssADN para la expresión de miARN fueron sintetizados por Roche y se comenzó realizando el anillamiento mezclando 10 µL de los cebadores directo y reverso (100 µM) ssADN con 20 µL de 2x Tampón de anillamiento (20 mM

Tris pH 8.0, 2 mM EDTA, 100 mM NaCl). Se incubaban durante 5 min a 100ºC en un baño de agua y se dejaban atemperar dentro del baño de agua durante toda la noche. A continuación, se procedía a la purificación de los cebadores dsADN anillados para reducir la concentración de sales, empleando el *kit* Qiaquick® PCR Purification (Qiagen) siguiendo las instrucciones del fabricante. Una vez anillados y purificados se llevaba a cabo la fosforilación de los cebadores dsADN como se indica en la tabla XX incubando durante 30 min a 37 ºC.

Compuestos	dsADN cebadores	Tampón 10X Promega T4 DNA ligase	Agua destilada	T4 PNK (10U/µL) T4 polynucleotide Kinase (Promega)
Volumen (µL)	1 µL	1 µL	7 µL	1 µL

Tabla XX. Mezcla de reacción para la fosforilación de los oligonucleótidos dsDNA.

Se realizaba por otro lado la digestión del vector pChlamiRNA2 con la enzima de restricción *SpeI* como se indica en la tabla XXI, incubando la digestión durante 1h a 37ºC. Para inactivar la acción de esta enzima se incubaba a 65ºC durante 20 min y a continuación la muestra se depositaba en hielo.

Compuestos	Vector pChlamiRNA2	3X Tampón H	Agua destilada	*SpeI* (10U/µL)
Volumen (µL)	10 µL	3 µL	16 µL	1 µL

Tabla XXI. Reacción de digestión del vector pChlamiRNA2.

El vector pChlamiRNA2 digerido se desfosforilaba añadiendo 2 µL SAP (1U/µL) (Shrimp Alkaline Phosphatase (Takara)), como se indica en la tabla XXII incubando 30 min a 37ºC. Para inactivar la enzima se incubaba 65ºC durante 15 minutos. A continuación, se precipitaba siguiendo las recomendaciones del fabricante de la SAP y se resuspendía en 20 µL de agua destilada.

Compuestos	Vector pChlamiRNA2	10X Tampón	Agua destilada	SAP (1U/µL)
Volumen (µL)	20 µL	5 µL	23 µL	1 µL

Tabla XXII. Reacción de desfosforilación del vector pChlamiRNA2.

Posteriormente, se ligaron el vector pChlamiRNA2 digerido con *SpeI* y defosforilado, con los cebadores dsADN anillados, fosforilados y purificados. Se realizaron dos ligaciones con diferentes diluciones de los cebadores dsADN (10x y 100x) empleando la ligasa T4 ADN de Promega como se indica en la tabla XXIII e incubando la reacción de ligación a Tº ambiente durante 1h.

Compuestos	Vector pChlamiRNA2 digerido y desfosforilado (20 ng/µL)	dsADN cebadores fosforilados (dilución 10X o 100X)	5X Tampón dilución ADN	Agua destilada	T4 ADN ligasa (3U/µL)
Volumen (µL)	1 µL	1 µL	1 µL	2 µL	0.5 µL

Tabla XXIII. Mezcla de reacción de ligación del vector pChlamiRNA2 y los oligonucleótidos dsADN.

Una vez realizadas las ligaciones, se llevó a cabo la transformación empleando células competentes como se explica en el apartado 2.4.11, empleando en este caso 100 µL de células competentes y 2.5 µL de reacción de ligación.

La selección de los clones correctos se realizó como describen Molnar y col., 2009. Para ello se picaban las colonias obtenidas y se realizaba una PCR de colonia empleando como cebador $precursor_{for}$: GCTAGAGGTGTTGGGTCGGTG y cebador $Spacer_{rev}$: TAGCGCTGATCACCACCACCC.

El programa de PCR utilizado para la comprobación de la obtención de clones correcto se muestra en la tabla XXIV.

Fases	HOT START	Desnaturalización		Anillamiento	Elongación		Mantenimiento
Temperatura (ºC)	95	95	95	65	72	72	14
Tiempo	30 (min)	5 (min)	30 (s)	30 (s)	30 (s)	7 (min)	Indefinido
		35 ciclos					

Tabla XXIV. Programa del termociclador para la comprobación de los clones de amiRNA.

Tras la PCR, si el resultado obtenido era la amplificación de una banda de 188 pb, el inserto estaba en la orientación correcta. Si no obteníamos amplificación nos indicaba que o no había inserto o estaba en la orientación contraria

Se llevó a cabo el aislamiento de ADN plasmídico de las colonias correctas como se indica en el punto 2.4.2. Y una vez obtenido el ADN plasmídico y como última comprobación para asegurar la inserción correcta del inserto se realizaba una doble digestión *XbaI/ EcoRI* como se explica en el apartado 2.4.9 corroborando el tamaño exacto del inserto.

Se realizaban Midiprep siguiendo las instrucciones del fabricante *(kit* PureYield™ Plasmid Midiprep System (Promega)*)* para aislar suficiente cantidad del plásmido para realizar las transformaciones y se linearizaba el vector pChlamiRNA2-ami RNA FT1 realizando una digestión con *HindIII* (Takara).

2.4.14.3 Transformación nuclear de *C.reinhardtii*.

Una vez obtenido el vector pChlamiRNA-amiARN FT1 linearizado se llevó a cabo la transformación nuclear de *C.reinhardtii* cc-425 arg2 cw15 por el método de perlas de vidrio como describe Kindle (1990). Se decidió realizar la transformación con el vector linearizado y sin linearizar.

Las células de *C.reinhardtii* cc-425 arg2 cw15 se crecieron en TAP suplementado con 200 mg L^{-1} de arginina (Arginina L, Sigma) hasta obtener una densidad celular de aproximadamente 2.5 x 10^6 células/mL. Obtenida la densidad celular deseada, las células se centrifugaron a Tª ambiente 5000 rpm, 5 min. Se realizaron 3 lavados con medio TAP sin arginina, para eliminar los restos de arginina del medio y se resuspendieron finalmente en TAP (1/100 de volumen original del cultivo) sin arginina, dejando las células aproximadamente a una concentración final de 10^8 células/mL. Las células se incubaron en este medio durante 3 h en agitación leve.

Para llevar a acabo la transformación nuclear, se mezclaron 300 µL de células, 300 mg de perlas de vidrio estériles (diámetro 0.45-0.52 mm Sigma), las cuales se lavaron con ácido HCl, se secaron y se esterilizaron en estufa (250°C durante 2-3 h), 100 µL 20% (p/v) polietilen glicol fresco (PEG 6000) y 1-2 µg de plásmido pChlamiRNA2-amiRNA FT1 (linearizado o sin linearizar) en tubos Falcon y se agiraron con un vortex 15 segundos a máxima velocidad (Fisher Vortex Genie II) . Las células transformadas se centrifugaron (5 min 1000 rpm) en un rotor basculante (Heraeus SPETECH Labofuge AC) y se plaquearon en placas con medio TAP sólido sin arginina. Después de 10 días de incubación a 25 ºC en un fitotrón con las mismas condiciones que los cultivos, aparecieron colonias individuales en las placas, que fueron posteriormente seleccionadas y analizadas.

2.4.14.4 Selección de líneas silenciadas para el gen *crft1*.

Después de varias semanas, las colonias que aparecieron en las placas resultantes de la transformación se picaban con palillos estériles y se pasaban a placas con medio TAP y medio mínimo HS 2 permitiendo el crecimiento individual de cada colonia. Ante la posibilidad de aparición de líneas afectadas en la actividad fotosintética, se seleccionaron estos dos medios, porque las líneas afectadas en fotosíntesis podrían crecer en medio TAP (ya que *Chlamydomonas* puede utilizar el acetato como fuente de carbono) pero no en medio mínimo.

Con las líneas silenciadas que crecían en ambos medios se realizaba aislamiento de ARN mediante Trizol como se detalla en el apartado 2.4.3. Tras el aislamiento del ARN se realizaba un tratamiento con DNAasa para limpiar de contaminación el ARN empleando el *kit* RQ1 RNAase Free DNase (Promega). Se partía de 100 µg de ARN aislado y se procedía como se indica en el Tabla XXV.

Volumen	Compuesto
5 µL	10x Tampón RQ1 RNase Free DNAse
5 µL	RQ1 RNase Free DNAse (1u/µg RNA)
X µL	H_2O
X µL	100 µg ARN aislado

↓

Incubación 37ºC durante 30 min

↓

5 µL RQ1 DNase Stop Solution

↓

Incubación a 65ºC durante 10 min para inactivar la DNase

↓

Volumen final 100 µL H_2O DEPC

Tabla XXV. Mezcla de reacción del tratamiento con DNAasa.

A continuación se procedía a la eliminación de las sales del tratamiento con DNasa empleando columnas del *kit* RNeasy Mini Kit (Quiagen) según las instrucciones del fabricante.

Con el ARN obtenido de las líneas silenciadas se realizaban PCRs, como se indica en el apartado 2.4.5, para comprobar la pureza del mismo y asegurarnos que no existía contaminación con ADN. Para ello se diseñaron cebadores en intrones de un gen implicado en fotosíntesis (Cebador directo: TGATCATAGTCTCCACCACG; Cebador reverso: ACGTGTCAGTAC ACCAGTTG).

Una vez seguros de la pureza del ARN de las líneas silenciadas se realizaba la síntesis de cDNA empleando el *kit* de Biorad realizando una mezcla de reacción con 4µL de 5x iScript reaction mix, 1 µL iScript reverse trascriptase, X µL agua libre de RNAasa y RNA XµL (1 µg RNA total) en un volumen final de 20 µL. Se incubaba en un termociclador: 5 min 25ºC, 30 min 42ºC, 5 min 85ºC y hold 4ºC.

Con el ADNc sintetizado se realizaban PCRs con diferentes cebadores (Tubulina y FT-exon 5-7) (Ver Tabla VII) para comprobar el grado de silenciamiento obtenido.

2.5 Técnicas electroforéticas.

2.5.1 Electroforesis en geles de agarosa.

Los fragmentos de ADN se separaron en función de su tamaño mediante electroforesis en geles horizontales de agarosa, con una concentración que variaba entre 1 y el 2 % (p/v) según el tamaño del fragmento de ADN que se quería resolver. Se utilizaba tampón TAE (40 mM Tris-HCl pH 7.6, 20 mM Ácido Acético y 1mM EDTA) (Sambrook, 1989).

Las muestras se mezclaban con tampón de carga (glicerol 60%, 0.5M EDTA pH 8.0, 35% agua y 25 mg de azul de bromofenol). El tamaño de los fragmentos se estimaba por su movilidad electroforética relativa usando como referencia marcadores estándar de peso molecular (1 Kb y 100 pb ladder Biotools).

2.5.2 Electroforesis en geles de acrilamida.

Las muestras protéicas se preparaban en tampón de carga 2x Laemmli y se incubaban a 95º C durante 10 min. El patrón polipeptídico de las muestras de extractos totales y cloroplastos aislados se estimaba por electroforesis en condiciones desnaturalizantes (en presencia de SDS) en geles verticales de acrilamida/bis acrilamida al 12% (Laemmli, 1970). Las electroforesis se llevaban a cabo en un equipo Mini-Protean III de Bio-Rad, siguiendo las instrucciones del fabricante. Se cargaba siempre la misma cantidad de clorofila o proteína por carril, tanto de los extractos celulares totales como de cloroplastos aislados y de fracciones estromáticas. Como referencia se corrían marcadores estándar de amplio rango (7- 200 KDa) de Bio-Rad. Una vez separadas las proteínas, los geles se teñían con Azul de Coomasie (0.2 % Coomasie Blue, 40 % Metanol y 10 % acético) o bien se electo-transferían a membranas de nitrocelulosa para la posterior detección inmunológica.

2.5.3 Ensayos de Inmunodetección (Western Blot).

Se realizaron transferencias a membranas de nitrocelulosa (Membrane Blotting, Pall Corporation). Una vez obtenidos los geles se lavaban durante 15 min en Tampón de Transferencia (25 mM Tris, 192 mM Glicina, 20% Metanol) para eliminar el exceso de SDS y se procedía a realizar la transferencia de proteínas a la membrana de nitrocelulosa en Tampón de Transferencia a 100 V durante 1 h.

Para la inmunodetección, en primer lugar, se bloqueaban las membranas 1 h a RT con 5 % (p/v) de leche desnatada en polvo en Tampón de Lavado (0.1 M Tris-HCl pH: 7.5, 0.5 M NaCl, 1 % (v/v) Tween-20). Se realizaban dos lavados de 10 min para eliminar restos del tampón de bloqueo. A continuación se incubaban con el anticuerpo primario (en 2 % (p/v) de leche desnatada en polvo en tampón de lavado) durante toda la noche con agitación orbital a 4ºC. Se realizaban dos lavados de 10 min para eliminar

restos del anticuerpo primario y se incubaban con el anticuerpo secundario acoplado a peroxidasa de rábano (en 2% de leche desnatada en polvo en Tampón de Lavado) durante 1 hora con agitación orbital a temperatura ambiente. Tras el tiempo de incubación se realizaban dos lavados de 5 min con tampón de lavado y se realizaba un último lavado de 10 min con agua destilada.

Se utilizaban diferentes anticuerpos primarios:

- anticuerpo policlonal de conejo contra la proteína CAH3 de *Chlamydomonas reinhardtii*, asociada a las membranas tilacoidales del cloroplasto (Villarejo y col., 2002). Este anticuerpo se utilizó para valorar el enriquecimiento de nuestras preparaciones de cloroplastos. Cedido por el profesor Göran Samuelsson de Umeå Plant Science Centre. Universidad de Umeå (Suecia).
- anticuerpo contra la proteína BiP de *Arabidopsis thaliana*, una chaperona asociada al retículo endoplásmico (ER) (Pedrazzini y col., 1997). Este anticuerpo se utilizó para valorar la contaminación con ER de nuestras preparaciones de cloroplastos. Cedido por el profesor Göran Samuelsson de Umeå Plant Science Centre. Universidad de Umeå (Suecia).
- anticuerpo policlonal de conejo contra residuos de fucosa en enlace $\alpha(1,3)$, cedido por el profesor Patrice Lerouge de la Universidad de Rouen (Francia).
- anticuerpo policlonal de conejo contra residuos de xilosa en enlace $\beta(1,2)$, cedido por el profesor Patrice Lerouge de la Universidad de Rouen (Francia).
- anticuerpo policlonal de conejo contra RuBisCo, que reconoce tanto la subunidad grande como la subunidad pequeña cedido por el profesor Göran Samuelsson de Umeå Plant Science Centre. Universidad de Umeå (Suecia).
- anticuerpo monoclonal FMG-1 que reconoce epítopos glucídicos asociados a las glicoproteínas flagelares. Cedido por el profesor Robert Bloodgood de la Universidad de Virginia (Estados Unidos).
- anticuerpo monoclonal de ratón contra hemoaglutinina (Santa Cruz).
- anticuerpo policlonal de conejo contra la proteína mtCA (carbónico anhidrasa mitocondrial) de *Chlamydomonas reinhardtii*. Este anticuerpo se utilizó para valorar la contaminación con mitocondrias de nuestras preparaciones de cloroplastos y la inducción del CCM en condiciones de bajo CO_2. Cedido por el profesor Göran Samuelsson de Umeå Plant Science Centre. Universidad de Umeå (Suecia).

El revelado se llevaba a cabo utilizando el reactivo ECL (Amersham) según las instrucciones del fabricante y películas de Rayos X (Amersham). También se realizaba el revelado empleando el *kit* Immun-Start ^{TM}Western C^{TM} Chemiluminescent (BioRad) en el sistema de imágenes de BioRad Molecular Imager Chemic Doc ^{TM} XRS (con el software Image Lab^{TM}) capaz de procesar imágenes de quimioluminiscencia, fluorescencia y colorimétricas.

2.5.4 Afinoblot.

- **Concanavalina A.**

La Concanavalina A (de Canavalia ensiformis (Frijol)) es una de las lectinas de plantas más empleadas que reconocen glicanos ricos en manosa. Y la peroxidasa (POX) es otra lectina que se une a Con A y nos permite revelar la existencia de glicoproteínas.

Las glicoproteínas que se unen a Concanavalina A (Con A), pueden ser localizadas en membranas de nitrocelulosa después de la transferencia electroforética desde geles de acrilamida. Las membranas de nitrocelulosa se incubaban secuencialmente con Con A y con Peroxidada (POX) que permite visualizar las muestras tras realizar revelados con diferentes reactivos.

Una vez transferidas las proteínas a la membrana de nitrocelulosa se bloqueaba durante toda la noche a 4ºC en TTBS (10 mM Tris-HCl pH 7.5, 0.5 M NaCl, 0.1% Tween 20). A continuación, se incubaba con 15 mL de Tampón Concavalina A (Con A) (10 mM Tris-HCl pH 7.5, 0.5 M NaCl, 0.1% Tween 20, 1 mM $CaCl_2$, 1 mM $MnCl_2$) más 15 µL de Con A (*Stock 1000X:* 25 mL/mL) durante 2 h a temperatura ambiente. Acto seguido se realizaban lavados de 15 min durante 1h con TTBS. Una vez realizados los lavados, se incubaba la membrana con 15 mL de TTBS más 3 µL de Peroxidasa (POX) (*Stock 1000x*: 50 mg/mL) durante 1h a temperatura ambiente. Se realizaban de nuevo cuatro lavados de 15 min y para finalizar un último lavado de 15 min con TBS (10 mM Tris-HCl pH 7.5, 0.5 M NaCl).

- **AAL (lectina *Aleuria Aurantia).***

Esta lectina se aisla de la seta *Aleuria Auranta*, es un dimero de dos subunidades idénticas de unos 36.000 daltons cada uno con un punto isoléctrico pH 9. Reconoce residuos de fucosa en enlace (1,6).

Una vez transferidas las proteínas a la membrana de nitrocelulosa se incubaba con tampón TTBS (0.1%), 1 mM CaCl2 y 1 mM MgCl2 junto con la lectina biotinilada AAL (Vector) (20 µg/mL) durante 1h 30 min a temperatura ambiente. Acto seguido se realizaban lavados de 10 minutos con TTBS. Una vez realizado los lavados, se incubaba la membrana con Streptavidina-HRP (Helthcare) (1:3000) durante 1h a temperatura ambiente. Para finalizar, se realizaban de nuevo tres lavados de 10 min con TTBS y se procedía al revelado.

El revelado se llevaba a cabo utilizando el reactivo ECL (Amersham) según las instrucciones del fabricante y películas de Rayos X (Amersham). También se relizaba el revelado empleando el *kit* Immun-Start ^{TM}Western C^{TM} Chemiluminescent (BioRad) en el sistema de imágenes de BioRad Molecular Imager Chemic Doc ^{TM} XRS (con el

software Image Lab™) capaz de procesar imágenes de quimioluminiscencia, fluorescencia y colorimétricas.

2.6 Técnicas Bioinformáticas

El genoma de *Chlamydomonas reinhardtii* ha sido recientemente secuenciado en su totalidad (Merchant y col., 2007) y está disponible en la dirección www.genome.jgi-psf.org/Chlre4/Chlre4.home.htmL. Utilizando este base de datos se llevó a cabo la búsqueda *in silico* de secuencias ortólogas de los distintos genes en el genoma de *C. reinhardtii*.

La búsqueda de secuencias de las distintas enzimas y genes de la ruta, pertenecientes a los distintos organismos usados en este estudio se realizó en las bases de datos del NCBI http://www.ncbi.nlm.nih.gov/gene/ y http://www.ncbi.nlm.nih.gov/protein/. Esta base de datos se usó además con las posibles secuencias de *Chlamydomonas* usando su aplicación http://blast.ncbi.nlm.nih.gov/Blast.cgi para buscar regiones de similitud con otras secuencias de función conocida.

Los alineamientos de las distintas secuencias se realizaron usando el paquete informático BioEdit,

La base de datos http://www.cazy.org/, se utilizó como apoyo para la descripción de las distintas familias, de los dominios funcionales etc, de las enzimas implicadas en la ruta. Se utilizó también el servidor SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html) para la predicción de dominios transmembrana.

IV. RESULTS

CHAPTER 1:

Evidence of N-glycoprotein transport to the chloroplast through the endomembrane system in *Chlamydomonas reinhardtii*.

The first aim of this work was to determine, in *Chlamydomonas reinhardtii*, the existence of the pathway for targeting N-glycoproteins to the chloroplast through the endomembrane system (ER and Golgi) as has been described in higher plants (Villarejo *et al*., 2005).

1.1 Purification of chloroplasts fractions from *Chlamydomonas reinhardtii*

Obtaining highly pure chloroplast fractions was essential to analyze the presence of glycoproteins in the chloroplast of *C.reinhardtii*. To achieve this purpose, we analyzed different chloroplast isolations obtained with two different methods in order to reduce contamination from mitochondrial and peroxisomal fractions in our samples. Contamination was tested by measuring Fumarase activity (mitochondria) or Hidroxypyruvate reductase activity (peroxisome) in total extracts and chloroplast fractions.

Our analysis revealed that the best yield and purest chloroplast preparations were obtained after a clumping-anticlumping treatment (see Materials and Methods, section 2.2.2) followed of isolation by loading onto two successive Percoll gradients which minimized mitochondrial contamination (see Table 1.1), although peroxisomal contamination was still higher than expected.

Sample	CO_2	Method/ gradient	HPR (nmol/mg chl min)		Fumarase (nmol/mg chl min)		Peroxisomes	Mitochondrias
			TE	CL	TE	CL		
C	H	Method 2 1 grad.	68.57	45.71	3671.4	1514.3	66 %	41 %
C	H	Method 2 2 grad.	91.86	45.71	4371.4	381.4	50 %	8 %

Table 1.1: Percentages of peroxisomes and mitochondria contamination in samples of chloroplasts (CL). Different isolates were performed using method 2 and measuring enzymatic activities with Fumarase as a marker of mitochondrias and Hydroxypyruvate reductase (HPR) as a marker of peroxisomes. H: High CO_2, TE: Total extract, CL: chloroplasts, HPR: Hidroxypyruvate reductase.

1.2 Presence of N-glycoproteins in the chloroplast of *C.reinhardtii.*

Once we established a good method for chloroplasts isolation, we analyzed the presence of glycoproteins in the chloroplast of *Chlamydomonas*. Chloroplast samples were analyzed using specific antibodies against β(1,2)-xylose and α(1,3)-fucose residues, because we know that these residues are typical in complex N-glycans of plants (Lerouge *et al.*, 1998).

In Figure 1.1A we observed that α(1,3)-fucose antibody recognized the presence of α(1,3)-fucose residues in proteins of total cell extract (TE), pure chloroplasts (CL) and stroma (STR) from wild type cells of *Chlamydomonas* growing in high CO_2 conditions. The protein pattern seems to be enriched in chloroplasts samples. In Figure 1.1B we observed that α(1,2)-xylose antibody recognized the presence of different α(1,2)-xylose residues in proteins of total cell extract (TE), pure chloroplasts (CL) and stroma (STR) from wild type cells of *Chlamydomonas* growing in high CO_2 conditions. As with fucose residues, the protein pattern seems to be enriched in chloroplasts samples. These results clearly indicate the presence of *N*-glycoproteins in the chloroplast of *Chlamydomonas* and that the N-glycan attached to the algal proteins harbors α(1,3)-fucose and β(1,2)-xylose residues.

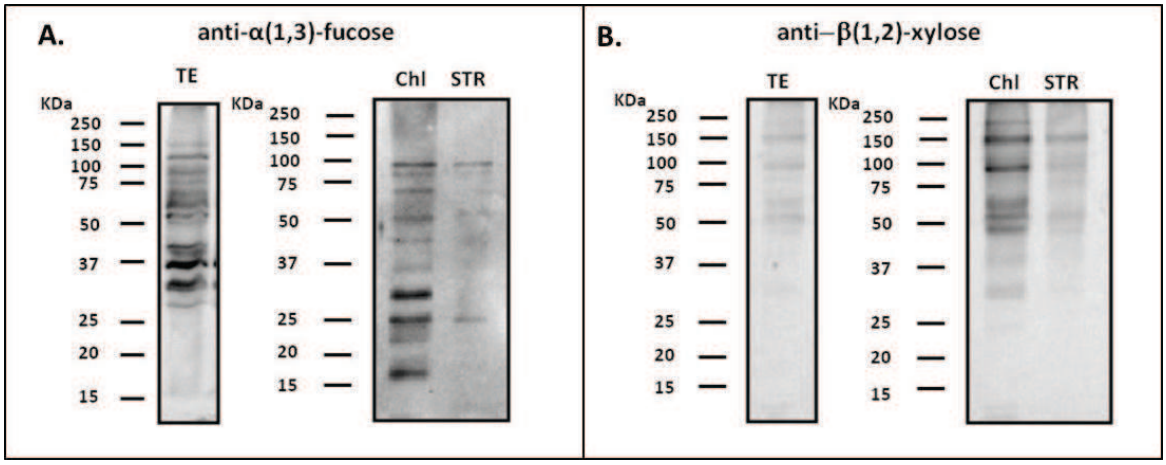


Figure 1.1: Glycosylation pattern of proteins in C.reinhardtii. 1 µg of chlorophyll of total cell extracts, chloroplast and stroma were separated by electrophoresis on 10% acrylamide gels and transference onto nitrocellulose membranes was made**. A.** Immunodetection was performed with a primary antibody raised against α(1,3)-fucose residues and a secondary antibody coupled to horseradish peroxidase. **B.** Immunodetection was performed with a primary antibody raised against β(1,2)-xylose residues and a secondary antibody coupled to horseradish peroxidase TE: Total extract, Chl: Chloroplasts, STR: Stroma.

To unravel the pathways involved in the transport of these plastid glycoproteins, total protein extracts and chloroplast fractions were obtained after treatment with different inhibitors of protein traffic through the endomembrane system (1 µM Monensin (M) for 2h; 25 µM Brefeldin A (BFA) for 24h; and 10 µg/mL Tunicamycin (T) for 24h) (See Materials and Methods, section 2.3.2). In addition to measuring Fumarase and HPR activities as has been already described, we performed an immunodetection with α-CAH3 antibody (Figure 1.2B) of total cells and chloroplasts from control and the 3 treatments fractions. CAH3 is a ***carbonic anhydrase exclusively located in*** thylakoid lumen. With this immunoblot we verified that our chloroplast preparations were highly enriched in this protein (Figure 1.2.B).

Immunodetection analysis with α(1,3)-fucose antibody (Figure 1.2A) showed that BFA, which blocks the retrograde transport of vesicles from the ER to Golgi prevents the arrival of glycoproteins at the plastid although glycoproteins are present in

total extracts. Tunicamycin, which inhibits glycosylation steps occurring in the ER, also prevents the arrival of fucosylated glycoproteins to the plastid.

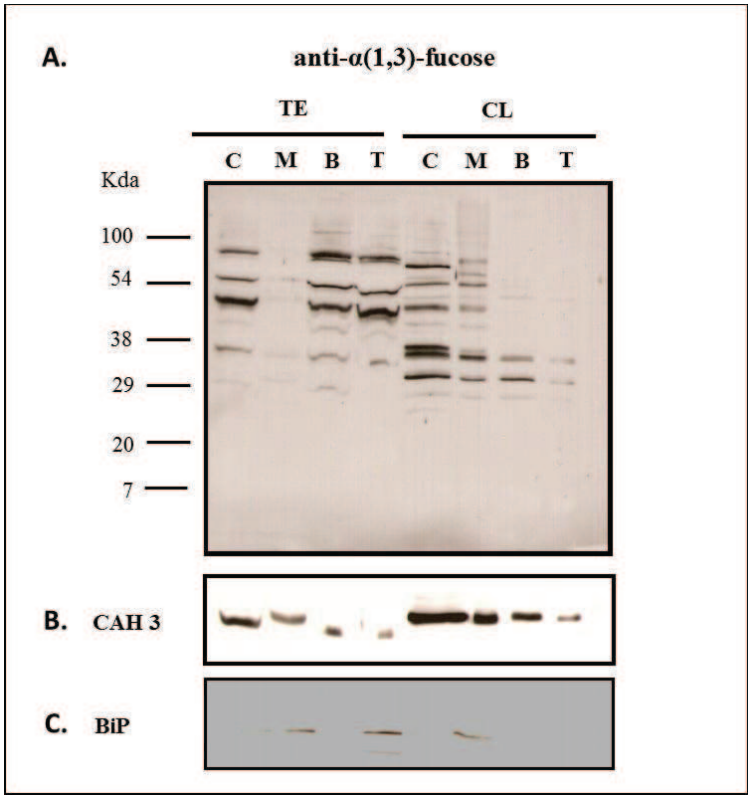


Figure 1.2: Glycosylation of proteins in chloroplasts and study the route of glycoproteins in C.reinhardtii. A. 1 µg of chlorophyll of total cell extracts, chloroplast and stroma were separated by electrophoresis on 10% acrylamide gels and transference onto nitrocellulose membranes was made**. A.** Immunodetection was performed with a primary antibody raised against α(1,3)-fucose residues and a secondary antibody coupled to horseradish peroxidase. **B**. Immunodetection was performed with a primary antibody raised against the protein CAH3 of Chlamydomonas reinhardtii and a secondary antibody coupled to horseradish peroxidase. **C.** Immunodetection was performed with a primary antibody raised against the protein BiP of Chlamydomonas reinhardtii and a secondary antibody coupled to horseradish peroxidase. TE: Total Extracts, CL:chloroplasts, C: Control, M: 1 µM Monensin, B: 25 µM Brefeldin A, T: 10 µg/mL Tunicamycin.

The results obtained with the samples treated with Monensin were not as conclusive as those of BFA or Tunicamycin. It has been described that Monensin is a monovalent ionophore, causing swelling of mature cisternae of plant Golgi apparatus (Boss *et al*., 1984). In the cells treated with Monensin it seemed that endomembrane fraction fractionated as a contamination of chloroplast fractions, as indicated by the presence of BiP chaperone, a protein involved in ER protein quality control. In addition these results indicate the occurrence of a route through the endomembrane system for proteins targeted to the chloroplast in this alga.

CHAPTER 2:

In silico and molecular dissection of the N-glycosylation biosynthetic pathway in *Chlamydomonas reinhardtii*

2.1 *In silico* analysis of *Chlamydomonas reinhardtii* genome revealed a set of genes encoding for proteins involved in the N-glycosylation pathway.

Once we proved the transport of N-glycoproteins to the chloroplast of *Chlamydomonas* through the endomembrane system (ER and Golgi) we did an *in silico* analysis searching for *C.reinhardtii* orthologs of enzymes involved in the biosynthesis of protein N-glycans.

Based on sequence homologies, we identified in the genome of *C.reinhardtii* a set of putative sequences that are involved in the different steps of the N-glycan biosynthesis and maturation in ER (Table 2.1). This *in silico* analysis for ER localized enzymes was performed mainly by the French group headed by Dr.Muriel Bardor and Dr. Patrice Lerouge (University of Rouen, France) of the ALGALGLYCO European consortium previously described in section II (Objective of this Work).

Most of the genes encoding for enzymes involved in the biosynthesis of dolichol pyrophosphate-linked oligosaccharide on the cytosolic face and in the lumen of the ER were identified in the genome of *C.reinhardtii* (Table 2.4). This bioinformatics work was done by sequence homology using human, *Arabidopsis* as well as others microalgae genes as templates and allowed us to identify in *Chlamydomonas* some of genes which encode proteins involved in the synthesis of the oligosaccharide precursor such as the Asparagine-linked-glycosylation genes (ALG) exception made of ALG3, ALG9, ALG12 and ALG 10 which don´t seem to be present in *Chlamydomonas* so far, or not in a form sharing common features with those of the organisms used as reference. However, we have found in *Chlamydomonas* N-glycan structures which would be consistent with the activity of these enzymes (see Chapter 5: Analysis of the N-glycan structure linked to endogenous algal glycoproteins).

In addition we have identified orthologs of the Oligosaccharyltransferase complex subunits (OST), responsible of the transfer of the oligosaccharide precursor onto the nascent glycoprotein. Enzymes involved in the early maturation steps in the endoplasmic reticulum or the Golgi apparatus, as well as the enzymes involved in protein quality control in the endoplasmic reticulum, have also been found.

All these data taken together indicate that in *Chlamydomonas* as it has been described for other eukaryotes (Banerjee et al., 2007) the ER localized machinery for N-glycan biosynthesis is well conserved.

Putative Function	Short Name	Protein id. (JGI v4.0)	Model name (JGI v4.0)	Gene location	Accession number (GenBank)	Prot length
ER cytosolic enzymes						
N-acetylglucosamine phosphostransferase	**CrALG 7**	CHLREDRAFT_396705	pasa_Sanger_mRNA14168	chromosome_16: 1990552-1993342	XP_001697886.1*	415
Beta-1,4-N-acetylglucosaminyl transferase	**CrALG 13**	CHLREDRAFT_182754	estExt_fgenesh2_kg.C_20123	chromosome_13: 3255029-3257117	XP_001693856.1	177
Beta-1,4-N-acetylglucosaminyl transferase	**CrALG 14**	CHLREDRAFT_397847http://www.genome.jp/dbget-bin/www_bget?cre+CHLREDRAFT_177895	pasa_Sanger_mRNA14329	chromosome_16: 3067735-3069662	XP_001699175.1*	222
Beta-1,4-mannosyl transferase	**CrALG 1**	CHLREDRAFT_108570 [1]	e_gwH.89.6.1	chromosome_12: 3825477-3826903	XP_001703220.1	475
Alpha-1,3-mannosyltransferase	**CrALG 2**	CHLREDRAFT_122195	e_gwW.62.8.1	chromosome_11: 1150916-1152886	XP_001701173.1	434
Alpha-1,2-mannosyltransferase	**CrALG 11**	CHLREDRAFT_19999	fgenesh1_pg.C_scaffold_51000063	scaffold_23: 188138-193311	XP_001700237.1	542
Dolichol-phosphate mannosyltransferase	**CrDPM 1**	CHLREDRAFT_138210	estExt_gwp_1W.C_270018	chromosome_3: 438314-439077	XP_001695552.1	254
Dolichol-phosphate glucosyltransferase	**CrALG 5**	CHLREDRAFT_18353	fgenesh1_pg.C_scaffold_39000051	chromosome_16: 679141-682477	XP_001698146.1	373
Flippase	**CrRFT**	CHLREDRAFT_178966	fgenesh2_pg.C_scaffold_62000004	scaffold_22: 349328-353741	XP_001701168.1	545
ER lumenal enzymes						
Alpha-1,3-glucosyltransferase	**CrALG 6**	CHLREDRAFT_36194	estExt_fgenesh1_pg.C_450055	chromosome_16: 5766199-5770411	XP_001699186.1	582
Alpha-1,3-glucosyltransferase	**CrALG 8**	CHLREDRAFT_104640 [1]	e_gwH.30.33.1	chromosome_9: 4322467-4325535	XP_001696729.1	518
Calnexin	**CrCLNX**	CHLREDRAFT_393004	pasa_Sanger_mRNA29881	chromosome_7: 6143630-6147986	XP_001703664.1	599
Calreticulin	**CrCLRT**	CHLREDRAFT_78954	estExt_GenewiseW_1.C_10142	chromosome_1: 5293813-5297125	XP_001689661.1	420
Glucosidase I	**CrGSI**	CHLREDRAFT_113687*[1]	e_gwW.2.301.1	chromosome_13: 2459268-2461989	XP_001693563	797
Glucosidase II, alpha-subunit	**CrGSIIA**	CHLREDRAFT_128630	estExt_gwp_1H.C_140194	chromosome_3: 4955490-4962655	XP_001692042.1	903
Glucosidase II, beta-subunit	**CrGSIIB**	CHLREDRAFT_381745	estExt_fgenesh1_pm.C_chromosome_170318	chromosome_17: 3554839-3558301	XP_001697152.1	486
Oligosaccharyltransferase complex subunits						
Dolichol-diphospho oligosaccharide--protein glycosyltransferase subunit	**CrDGL 1**	CHLREDRAFT_121156	e_gwW.43.13.1	chromosome_14: 987242-990915	XP_001699044.1	424
Ribophorin I	**CrRPN1**	CHLREDRAFT_131340	estExt_gwp_1H.C_310084	chromosome_12: 4550885-4555389	XP_001697027	453
Ribophorin II	**CrRPN2**	CHLREDRAFT_175668	fgenesh2_pg.C_scaffold_29000141	chromosome_8: 1593746-1596364	XP_001696147.1	262
dolichol-diphospho oligosaccharide--protein glycosyltransferase subunit	**CrDAD 1**	CHLREDRAFT_141763	Chlre2_kg.scaffold_5000173	chromosome_2: 4617959-4619399	XP_001699953	108
dolichol-diphospho oligosaccharide--protein glycosyltransferase subunit	**CrSTT3B**	CHLREDRAFT_127991	estExt_gwp_1H.C_100286	chromosome_7: 2282859-2291158	XP_001690442.1	716

dolichol-diphospho oligosaccharide--protein glycosyltransferase subunit	**CrSTT3A**	CHLREDRAFT_148751	Chlre2_kg.scaffold_24000150	chromosome_2: 6262471-6270238	XP_001695034.1	424
dolichol-diphospho oligosaccharide--protein glycosyltransferase subunit	**CrOST3**	CHLREDRAFT_196082	SAN_chlre3.45.14.2.11	chromosome_1: 8748501-8751480	**No correspondance in GenBank**	

Table 2.1: References of predicted proteins involved in the N-glycosylation pathway and quality control of secreted proteins in C. reinhardtii. Translated products have been deduced from sequences identified in the version 4.0 (http://genome.jgi-psf.org/Chlre4/Chlre4.info.htmL) of the genome except those which are noted with an asterik (*) which have been identified in the version 3.0; 1partial sequence on the amino end.

In this work we focused mainly on the Golgi localized glycosyl hydrolases and glycosyltranferases, involved in the final maturation of the protein-linked N-glycans in *Chlamydomonas reinhardtii*, since these are essential to determine the characteristic N-complex glycan structure of each organism. In regard, to Golgi enzymes involved in N-glycan biosynthesis, *in silico* analyses of the *C.reinhardtii* genome revealed the presence of genes encoding for a putative α1,2-Mannosidase I (CrManI), α-Mannosidase II (CrManII), α1,3-fucosyltransferase (CrFT1) and β1,2-xylosyltransferase (CrXylT). But, we didn´t find genes encoding for putative N-Acetylglucosaminyltransferase I (GntI) and N-Acetylglucosaminyltransferase II (GntII) (Table 2.2).

Putative Function	Short Name	Protein id. (JGI v4.0)	Model name (JGI v4.0)	Gene location	Accession number (GenBank)	Prot length
Golgi enzymes						
Alpha-1,2-mannosidase I	**CrManI**	CHLREDRAFT_178232	fgenesh2_pg.C_scaffold_50000038	chromosome_7: 3304731-3311367	XP_001700094.1	670
Mannosidase II	**CrManII**	CHLREDRAFT_144252	Chlre2_kg.scaffold_10000132	chromosome_10: 2650649-2659824	XP_001690386.1	1360
Beta- 1,2-xylosyltransferase	**CrXylT**	CHLREDRAFT_191368	estExt_fgenesh2_pg.C_240262	chromosome_2: 6833318-6837848	XP_001695075.1	522
Alpha-1,3-fucosyltransferase	**CrFT1**	CHLREDRAFT_191875	estExt_fgenesh2_pg.C_250145	scaffold_31: 155613-159255	XP_001695259.1	407

Table 2.2: Predicted proteins involved in the Golgi N-glycosylation pathway in C. reinhardtii. Translated products have been deduced from sequences identified in the version 4.0 of the genome (http://genome.jgi-psf.org/Chlre4/Chlre4.info.html).

2.1.1 Identification of α1,2-Mannosidase (CrManI) ortholog in *C.reinhardtii.*

α-Mannosidases have been classified into two groups based on amino acid sequence homology and on biochemical properties (Daniel *et al*., 1994; Moremen *et al.,* 1994; Tremblay and Herscovics, 1999). Class I α-Mannosidases (ManI) specifically hydrolyze

α(1,2)-linked mannose residues and in contrast, Class II α-Mannosidase (ManII) can cleave α(1,2)-, α(1,3)-, α(1,6)-linked mannose residues.

α(1,2)-Mannosidases I belong to glycosyl hydrolase family 47 of the *Carbohydrate Active Enzyme* database (http://www.cazy.org) annotated in Pfam database as PF01532 (http://pfam.sanger.ac.uk/). These enzymes are inverting glycosyl hydrolases that are highly specific for α(1,2)-mannose residues, require Ca^{2+} for catalytic activity and are sensitive to inhibition by pyranose analogs such as 1-deoxymannojirimycin and kifunensine (Lipari *et al*., 1995; González *et al.,* 1999). Class I α-Mannosidases are conserved through eukaryotic evolution and do not share sequence homology with class II α-mannosidases, such as Golgi α -Mannosidase II or the catabolic lysosomal and cytoplasmic α-mannosidases (González *et al*., 1999; Herscovics, 2001; Liebminger *et al*., 2009).

The Class I α-Mannosidases play several roles in the oligosaccharide-trimming reactions in the ER and Golgi (Daniel *et al*., 1994; Herscovics, 1999). Three subfamilies of mammalian Class I α-Mannosidases have been identified; the ER mannosidase I subfamily cleaves a single residue from $Man_9GlcNAc_2$ to generate the $Man_8GlcNac_2$ structure; the Golgi Mannosidase I subfamily cleaves $Man_{9\text{-}8}GlcNAc_2$ structures to $Man_5GlcNAc_2$ and the EDEM subfamily of α-mannosidase-related proteins does not appear to have an intrinsic hydrolase activity but seem to be required for disposal of terminally misfolded glycoproteins in ER-associated degradation (ERAD) (Tempel *et al*., 2004).

The Golgi α-Mannosidases I subfamily of proteins are type II membrane proteins localized in the early Golgi compartment and catalyze the trimming of the four α(1,2)-mannose residues. These enzymes contain three highly conserved amino acid sequences that are required for the catalytic activity at their C-termini (Tremblay and Herscovics, 1999; Mast and Moremen, 2006).

α-Mannosidase I from *Droshopila melanogaster* (Kerscher *et al.,* 1995), *Penicillium citrium* (Yoshida and Ichishima, 1995) and *Aspergillus saitoi* (Inoue *et al*., 1995) have been cloned and have similar amino acid sequences to yeast and mammalian enzymes (Lipari and Herscovics, 1996).

CrManI candidate presents 37% of sequence identity with *Arabidospis thaliana*; 35% with *Droshopila melanogaster* and 51% of identity with *Homo sapiens* (Figure 2.1B). The sequence exhibits the appropriate type II membrane protein topology with a transmembrane domain of 23 residues, located right in the beginning of N-terminus from residue 1 to 23 (MGSLALLTYVWVVFVLHPARVAD) predicted by SOSUI database server for secondary structure prediction of membrane proteins (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html). The putative α(1,2)-mannosidase I (CrMan I) of *C.reinhardtii* exhibits the three conserved catalytic motifs of α-Mannosidases I, the Threonine (T) residue of the motif III, and the two cysteine

residues (Cys-427 and Cys-462) essential for the mannosidase activity (Tempel *et al.*, 2004; Kajiura *et al.,* 2010) (Figure 2.1B).

The gene encoding for the putative CrmanI presents 15 exons and 14 introns (Figure 2,1A).

A.

B.

```
CrManI   1   ----MGSLALLTYVWVVFVLHPARVADAAVTQGGADSGGARIGRLR--LNTGGATGHDLSHLLFQTP---------- 61
AtManI   1   ------------------------------------------------------------------------------ 1
DmManI   1   -------MKDGGHITLTLPPGSAATTDE-RGRKSLRRAWNQLPRCQRNLIILGITGFCVTVLLCLSGDQLLAASLKDVDT 72
HsManI   1   MYPPPPPPPPHRDFISVTLSFGESYDNSKSWRRRSCWRKWKQLSRLQRNMILFLLAFLLFCGLLFYIN---LADHWKALAF 77

CrManI   61  ------------------SHPTKPQKTAAAKASSPPPPPPDEDDVPEEGPHPMRHYPYQDAKYRIWSLPKEDVSPRCRASG 123
AtManI   1   ------------------------------------MSKSPNESGLNEKGKVTFVGGLRLGG--LLRKPPRLPPRLSPDEG 43
DmManI   73  KAEVAAYQMPLPNPHHHPGVDKDVPKDTAAVPVPADKRAPRVEAVSDKFNELVAYGEANSAENVARLPETVAAEGLPQAS 152
HsManI   78  RLEEEQ--------KMRPEIAGLKPANPPVLPAP-QKADTDPENLPEISSQKTQRHIQRGPPHLQIRPPSQDLKDGTQEE 148

CrManI   124 ICDGKYDCGADGMGCIT----------------------------------------------------------DA 142
AtManI   44  QLRGSSTNGSTISNSDP----------------------------------------------------------KW 62
DmManI   153 AMAVALPPPDSNKEQDIGGIDILNNVEEPVAQDPAEPLSEGEQLEQAIKNPLTLGGVSFKEHLKKIKPILAKGQHFHGAT 232
HsManI   149 ATKRQEAPVDPRPEGDP---------QRAVISWRGAVIEP----EQGTELPPRRAEVPTKPPLP---PARTQGTPVH--L 210

CrManI   143 LKRKMKVRDAAAWTWKGYRQYAWGHDELNAGSRNSREWFN-MGLTIVDSLDTLQILGLLEEYAEARYWVVNHLD---LNQ 218
AtManI   63  AARQQSVKEAFDHAWSGYRKYAMGYDELMPISQKGVDGLGGLGATVVDALDTAMIMGLDNIVSEAGSWVETHLLERISQK 142
DmManI   233 NERQSAVVAAFKHSWAGYKKYAWGHDNLKPISQYSHEWFG-LGLTIVDSLDTMYIMGLDDEFKEGRDWVEQSL--RFDTK 309
HsManI   211 NYRQKGVIDVFLHAWKGYRKFAWGHDELKPVSRSFSEWFG-LGLTLIDALDTMWIIGLRKEFEEARKWVSKKL--HFEKD 287

CrManI   219 GDVSVFETTIRILGGLLAAAFYHSGGDE-------------AELMKAVEFADRLMPAFNT-SSGLPQPHFSVHDKNVATYR 284
AtManI   143 GQVNLFETTIRVLGGLLSAYHLSGGEQGTVNMTHVGPKPVIYLNIAKDLADRLLSAFTSSPTPVPFCDVILHESTAHPAP 222
DmManI   310 RDVNLFEVTIRVLGGLLSAYHLSGDT--------------MGLAKAAELGNRLLPAFQS-PSNIPYSDVNLGDLSAHSPK 374
HsManI   288 VDVNLFESTIRILGGLLSAYHLSGDS-------------LELRKAEDFGNRLMPAFRT-PSKIPYSDVNIGTGVAHPPR 352

CrManI   285 GSAGSSCLAEIGTLSMEFTAVSRLSGRPEFRDTAMKTWPIIKSMP-SMDGLYCTMLVADLLSCNG-NHYTFGASADSTYE 362
AtManI   223 GG--ASSTAEVASVQLEFNYLSSISGDPKMSTEAMKVLAHIKTLP-KTEGLVPIYISPQTGDFVG-ENIRLGSRGDSYYE 298
DmManI   375 WSP-DSSTSEVTTIQLEFRDLSRSTNISIMEQVAHKVNEKVHDLE-KNHGLVPIFINANTGTFRNYATISLGARGDSYYE 452
HsManI   353 WTS-DSTVAEVTSIQLEFRELSRLTGDKKFQEAVEKVTQHIHGLSGKKDGLVPMFINTHSGLFTHLGVFTLGARADSYYE 431

CrManI   363 YMLKQWVLTNGTDEMCLDMYKKAIGGMRRHMLTELWMGPDIGEVWIAAENDAGAS--RNLVLEHLTGFLPGTMALGHMYG 440
AtManI   299 YLIKVWLQQGAKLNSNFTYLHDMYIEAMKGVRHLLVQNSIPKGLVFVGELPYGSKGEFSPKMDHLVCFLPGILALGATKG 378
DmManI   453 YLLKQWIQTGRKDN---DNLILDYMQAVDGVLTQLMRRTPREHWVYIGELINGKD--FKPKMDHLTGYLPGTLILGHQNG 527
HsManI   432 YLLKQWIQGGKQE----TQLLEDYVEAIEGVRTHLLRHSEPSKLTFVGELAHGR---FSAKMDHLVCFLPGTLALGVYHG 504

CrManI   441 VNTG--------GAGEDDDLMVAIKLMKACYELYHQTPSGVAWDSVHLDEPVEVTSTREAGVIDSDDGDDGADGVDGLGY 512
AtManI   379 LTKEQALKENLLSFEDLENLKTAEDLAKTCFEMYEVTATGLAPEIAYFHTKDYTEDGLDG---------------------- 438
DmManI   528 MPDS--------------HLILARDLLDTCYQTYMMNPTHLAAEISYFALTEKDDQ----------------------- 569
HsManI   505 LPAS--------------HMFLAQELMETCYQMNRQMETGLSPEIVHFNLYPQPGRR----------------------- 547

CrManI   513 VVLDAEERRHLMAEASGGAGDGAKPPGWGKVKYEFAFRSREVFLREEVAESLFYLWRATGDPIYREWGWNMFRAYERWCR 592
AtManI   438 ----------------------GNKSSMYANDIIIKEADRHNLLREETVESLFVLYRITKDTKYRDQGWQIFEAFEKYTK 496
DmManI   569 ------------------------------DIYVKENDAHNLLREEFVESLYYFYSITGNRTYQDMGWKIFQAFETHAK 618
HsManI   547 ------------------------------DVEVKEADRHNLLREETVESLFYLYRVTGDRKYQDWGWEILQSFSRFTR 596

CrManI   593 VDSG----------------GYQMESFWIAETLKYFYLLFSDDPNEIPLDEFVFNTEAHPLAIWGSATDRKLRKILERAF 656
AtManI   497 VKSGGYTSLDDVTEVPPHR-RDKMETFFLGETLKYLYLLFGDD-SVIPLDKFVFNTEAHPLPIRRNT------------ 561
DmManI   619 VNAG-YTSMGNVKNTQSTRLRDLMESFWMSETLKYFYLLFSDDRKEIDLEQWVFNSEGHPLPVYPLKT------------ 685
HsManI   597 VPSGGYSSINNVQDPQKPEPRDKMESFFLGETLKYLYLLFSDDPNILSLDAYVFNTEAHPLPIWTPA------------ 663

CrManI   657 YAFLMHQHFRNDAL 670
AtManI   561 -------------- 561
DmManI   685 -------------- 685
HsManI   663 -------------- 663
```

Figure 2.1: α1,2-mannosidases I of Family GH47. **A.** ***Arrangement of the potential crmanI gene with 15 exons and 14 introns.*** Blue boxes are exons and arrows indicate primers designed to PCR amplification. **B.** ***Aminoacid sequence alignment of the Chlamydomonas CrManI.*** The aminoacid sequences containing the conserved cysteine residues (*) are shown from *C.reinhardtii* (CrManI), *A.thaliana* (AtManI), *D.melanogaster* (DmManI) and *Homo sapiens* (HsManI). The additional cysteine residues in *Chlamydomonas* α(1,2)-mannosidase I are marked with an arrow. The conserved class I α(1,2)-mannosidase motifs are indicated by red underlines.

2.1.2. Identification of a Mannosidase II (CrManII) ortholog in *C.reinhardtii.*

Mannosidases II (ManII) are glycosyl hydrolases that reside in the Golgi apparatus of eukaryotes and play a key role in the N-linked glycosylation of proteins (Moremen, 2002; Chui *et al.,* 1997). Mannosidases II have a high degree of sequence conservation among many eukaryotes. It has been classified as family 38 of glycosyl hydrolases of the *Carbohydrate Active Enzyme* database and catalyse the removal of two mannose residues (α-(1,3)-linked and α–(1,6)-linked mannoses) from GlcNAcMan5GlcNAc2, previous to form the core GlcNAc2Man3GlcNAC2 glycosyl structure, which is the committed step of complex N-glycan synthesis in most organisms (See Figure II Introduction Section 2.2) (Henrissat, 1991; Henrissat and Bairoch, 1993). The reaction requires the presence of the terminal GlcNAc added to the glycan by N-acetylglucosaminyltransferase I (Gnt I) in the non-branched antenna.

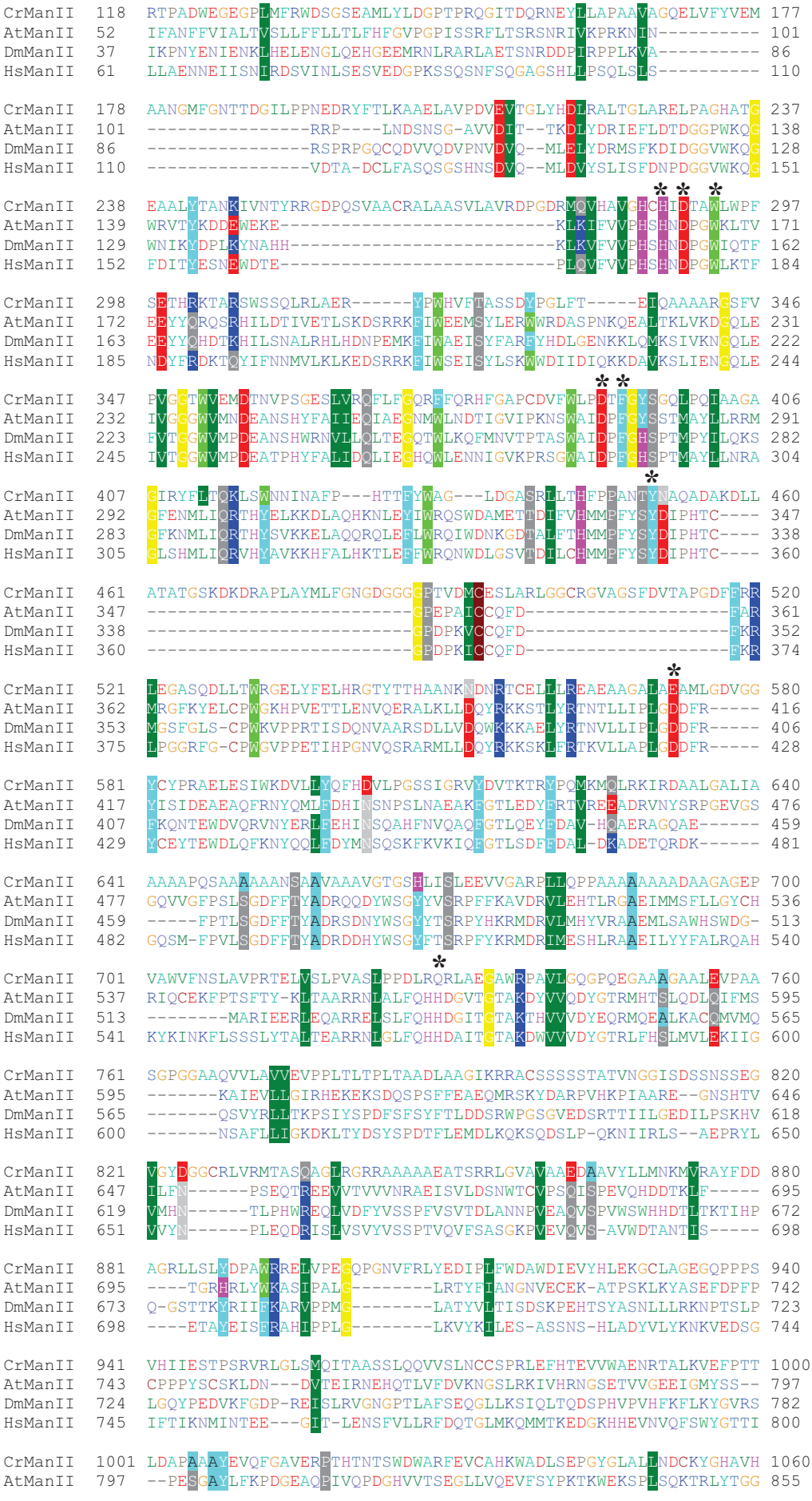
In silico analysis of *C.reinhardtii* genome revealed the presence of one potential gene encoding for a putative α-mannosidase II (CrManII). This putative gen *crmanII* presents 21 exons and 20 introns (Figure 2.2A). The putative CrManII protein presents 34% of sequence identity with *Arabidopsis thaliana*; 35% with *Droshopila melanogaster* and 50% of identity with *Homo sapiens* (Figure 2.2B). CrManII putative protein does not exhibit a clear type II membrane protein topology, since the SOSUI server prediction for a transmembrane domain in the N-terminus did not completely fulfill the hydrophobicity ratio to be considered a safe prediction. CrManII in *C.reinhardtii* is a large protein containing the three typical Pfam domains of GH38 CAZy family of glycosyl hydrolases: the N-terminal domain (PF01074), middle domain (PF09261) and C-terminal domain (PF07748). Data derived from crystal structure of *D.melanogaster* ManII suggest that some conserved residues are involved in the coordination of the zinc ion required for catalysis and in the formation of the active site (van den Elsen *et al*., 2001). These residues are also conserved in *Chlamydomonas reinhardtii* (Figure 2.2B).

A.

B.

```
CrManII   1    MRPASGGSGAAGPAAPAAGAAPAVSDVPGRWVPQHHRDITLGRLDGFLAAGQFADVNLRA 60
AtManII   1    ------------------------------------------------------MPFSS 5
DmManII   1    ------------------------------------------------------MLRIR- 5
HsManII   1    ------------------------------------------------------MKLS- 4

CrManII   61   AMWRRRSADAVSLEVYSHPKDVPYPPYDVAIKMPYRPAAVG---EAFGPSWTTHWFRVRA 117
AtManII   6    YIGNSRRSSTGGGTGGWGQSLLPTALSKSKLAINRKPRK--------------RTLVVNF 51
DmManII   5    ----RRFALVICSGCLLVFLSLYIILN---FAAP----------------------AATQ 36
HsManII   4    ----RQFTVFGSAIFCVVIFSLYLMLDRGHLDYPRNPRREGSFPQGQLSMLQEKIDHLER 60
```


CrManII 118 RTPADWEGEGPLMFRWDSGSEAMLYLDGPTPRQGITDQRNEYLLAPAAVAGQELVFYVEM 177
AtManII 52 IFANFFVIALTVSLLFFLLTLFHFGVPGPISSRFLTSRSNRIVKPRKNIN---------- 101
DmManII 37 IKPNYENIENKLHELENGLQEHGEEMRNLRARLAETSNRDDPIRPPLKVA---------- 86
HsManII 61 LLAENNEIISNIRDSVINLSESVEDGPKSSQSNFSQGAGSHILPSQLSLS---------- 110
CrManII 178 AANGMFGNTTDGILPPNEDRYFTLKAAELAVPDVEVTGLYHDLRALTGLARELPAGHATG 237
AtManII 101 ----------------RRP----LNDSNSG-AVVDIT--TKDLYDRIEFLDTDGGPWKQG 138
DmManII 86 ----------------RSPRPGQCQDVVQDVPNVDVQ--MLEIYDRMSFKDIDGGVWKQG 128
HsManII 110 ----------------VDTA-DCLFASQSGSHNSDVQ--MLDVYSLISFDNPDGGVWKQG 151
CrManII 238 EAALYTANKIVNTYRRGDPQSVAACRALAASVLAVRDPGDRMQVHAVGHCHIDTAWLWPF 297
AtManII 139 WRVTYKDDEWEKE----------------------------KLKIFVVPHSHNDPGWKLTV 171
DmManII 129 WNIKYDPLKYNAHH----------------------------KLKVFVVPHSHNDPGWIQTF 162
HsManII 152 FDITYESNEWDTE----------------------------PLQVFVVPHSHNDPGWLKTF 184
CrManII 298 SETHRKTARSWSSQLRLAER------YPWHVFTASSDYPGLFT-----EIQAAAARGSFV 346
AtManII 172 EEYYQRQSRHILDTIVETLSKDSRRKFIWEEMSYLERWWRDASPNKQEALTKLVKDGQLE 231
DmManII 163 EEYYQHDTKHILSNALRHLHDNPEMKFIWAEISYFARFYHDLGENKKLQMKSIVKNGQLE 222
HsManII 185 NDYFRDKTQYIFNNMVLKLKEDSRRKFIWSEISYLSKWWDIIDIQKKDAVKSLIENGQLE 244
CrManII 347 PVGGTWVEMDTNVPSGESLVRQFLFGQRFFQRHFGAPCDVFWLPDTFGYSGQLPQIAAGA 406
AtManII 232 IVGGGWVMNDEANSHYFAIIEQIAEGNMWLNDTIGVIPKNSWAIDPFGYSSTMAYLLRRM 291
DmManII 223 FVTGGWVMPDEANSHWRNVLLQLTEGQTWLKQFMNVTPTASWAIDPFGHSPTMPYILQKS 282
HsManII 245 IVTGGWVMPDEATPHYFALIDQLIEGHQWLENNIGVKPRSGWAIDPFGHSPTMAYLLNRA 304
CrManII 407 GIRYFLTQKLSWNNINAFP---HTTFYWAG---LDGASRLLTHFPPANTYNAQADAKDLL 460
AtManII 292 GFENMLIQRTHYELKKDLAQHKNLEYIWRQSWDAMETTDIFVHMMPFYSYDIPHTC---- 347
DmManII 283 GFKNMLIQRTHYSVKKELAQQRQLEFLWRQIWDNKGDTALFTHMMPFYSYDIPHTC---- 338
HsManII 305 GLSHMLIQRVHYAVKKHFALHKTLEFFWRQNWDLGSVTDILCHMMPFYSYDIPHTC---- 360
CrManII 461 ATATGSKDKDRAPLAYMLFGNGDGGGPTVDMCESLARLGGCRGVAGSFDVTAPGDFFRR 520
AtManII 347 ------------------------GPEPAICCQFD--------------------FAR 361
DmManII 338 ------------------------GPDPKVCCQFD--------------------FKR 352
HsManII 360 ------------------------GPDPKICCQFD--------------------FKR 374
CrManII 521 LEGASQDLLTWRGELYFELHRGTYTTHAANKNDNRTCELLIREAEAAGALAEAMLGDVGG 580
AtManII 362 MRGFKYELCPWGKHPVETTLENVQERALKLLDQYRKKSTLYRTNTLLIPLGDDFR----- 416
DmManII 353 MGSFGLS-CPWKVPPRTISDQNVAARSDLLVDQWKKKAELYRTNVLLIPLGDDFR----- 406
HsManII 375 LPGGRFG-CPWGVPPETIHPGNVQSRARMLLDQYRKKSKLFRTKVLLAPLGDDFR----- 428
CrManII 581 YCYPRAELESIWKDVLLVQFHDVLPGSSIGRVYDVTKTRYPQMKMQLRKIRDAALGALIA 640
AtManII 417 YISIDEAEAQFRNYQMLFDHINSNPSLNAEAKFGTLEDYFRTVREEADRVNYSRPGEVGS 476
DmManII 407 FKQNTEWDVQRVNYERLFEHINSQAHFNVQAQFGTLQEYFDAV-HQAERAGQAE------ 459
HsManII 429 YCEYTEWDLQFKNYQQLFDYMNSQSKFKVKIQFGTLSDFFDAL-DKADETQRDK------ 481
CrManII 641 AAAAPQSAAAAAANSAAVAAAVGTGSHLISLEEVVGARPLLQPPAAAAAAAADAAGAGEP 700
AtManII 477 GQVVGFPSLSGDFFTYADRQQDYWSGYYVSRPFFKAVDRVLEHTLRGAEIMMSFLLGYCH 536
DmManII 459 -----FPTLSGDFFTYADRSDNYWSGYYTSRPYHKRMDRVLMHYVRAAEMLSAWHSWDG- 513
HsManII 482 GQSM-FPVLSGDFFTYADRDDHYWSGYFTSRPFYKRMDRIMESHLRAAEILYYFALRQAH 540
CrManII 701 VAWVFNSLAVPRTELVSLPVASLPPDLRQRLAEGAWRPAVLGQGPQEGAAAGAALEVPAA 760
AtManII 537 RIQCEKFPTSFTY-KLTAARRNLALFQHHDGVTGTAKDYVVQDYGTRMHTSLQDLQIFMS 595
DmManII 513 --------MARIEERLEQARRELSLFQHHDGITGTAKTHVVVDYEQRMQEALKACQMVMQ 565
HsManII 541 KYKINKFLSSSLYTALTEARRNLGLFQHHDAITGTAKDWVVVDYGTRLFHSLMVLEKIIG 600
CrManII 761 SGPGGAAQVVLAVVEVPPLTLTPLTAADLAAGIKRRACSSSSSTATVNGGISDSSNSSEG 820
AtManII 595 -------KAIEVLLGIRHEKEKSDQSPSFFEAEQMRSKYDARPVHKPIAARE--GNSHTV 646
DmManII 565 -------QSVYRLLTKPSIYSPDFSFSYFTLDDSRWPGSGVEDSRTTIILGEDILPSKHV 618
HsManII 600 -------NSAFLLIGKDKLTYDSYSPDTFLEMDLKQKSQDSLP-QKNIIRLS--AEPRYL 650
CrManII 821 VGYDGGCRLVRMTASQAGLRGRRAAAAAAEATSRRLGVAVAAEDAAVYLLMNKMVRAYFDD 880
AtManII 647 ILFN------PSEQTREEVVTVVVNRAEISVLDSNWTCVPSQISPEVQHDDTKLF----- 695
DmManII 619 VMHN------TLPHWREQLVDFYVSSPFVSVTDLANNPVEAQVSPVWSWHHDTLTKTIHP 672
HsManII 651 VVYN------PLEQDRISLVSVYVSSPTVQVFSASGKPVEVQVS-AVWDTANTTS----- 698
CrManII 881 AGRLLSLVDPAWRRELVPEGQPGNVFRLYEDIPLFWDAWDIEVYHLEKGCLAGEGQPPPS 940
AtManII 695 ----TGRFRLYWKASIPALG--------LRTYFIANGNVECEK-ATPSKLKYASEFDPFP 742
DmManII 673 Q-GSTTKYRIIFKARVPPMG--------LATYVLTISDSKPEHTSYASNLLLRKNPTSLP 723
HsManII 698 ----ETAYEISFRAHIPPLG--------LKVYKILES-ASSNS-HLADYVLYKNKVEDSG 744
CrManII 941 VHIIESTPSRVRLGLSMQITAASSLQQVVSLNCCSPRLEFHTEVVWAENRTALKVEFPTT 1000
AtManII 743 CPPPYSCSKLDN---DVTEIRNEHQTLVFDVKNGSLRKIVHRNGSETVVGEEIGMYSS-- 797
DmManII 724 LGQYPEDVKFGDP-REISLRVGNGPTLAFSEQGLLKSIQLTQDSPHVPVHFKFLKYGVRS 782
HsManII 745 IFTIKNMINTEE---GIT-LENSFVLLRFDQTGLMKQMMTKEDGKHHEVNVQFSWYGTTI 800
CrManII 1001 LDAPAAAYEVQFGAVERFTHTNTSWDWARFEVCAHKWADLSEPGYGLALLNDCKYGHAVH 1060
AtManII 797 --PESGAYLFKPDGEAQPIVQPDGHVVTSEGLLVQEVFSYPKTKWEKSPLSQKTRLYTGG 855

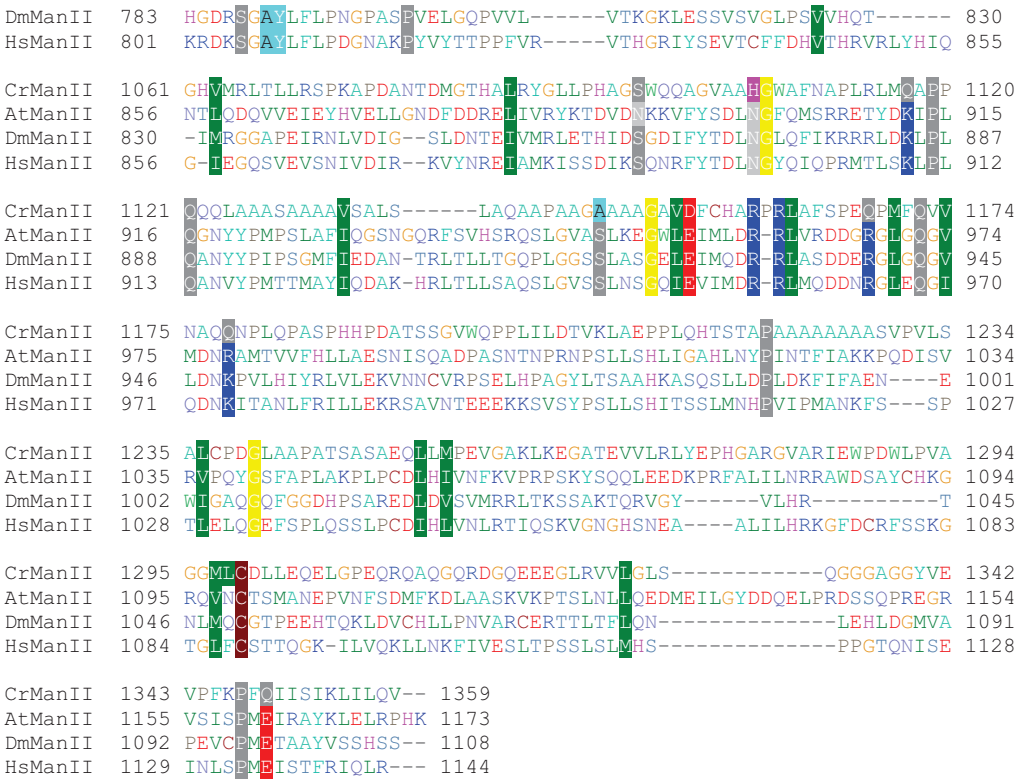


Figure 2.2: α-mannosidases II of family GH38*. A. *Arrangement of the potential crmanII gene with 21 exons and 20 introns. Blue boxes are exons and arrows indicate primers designed to PCR amplification. **B. *Aminoacid sequence alignment of the Chlamydomonas CrManII.*** Identical aminoacids in the mannosidasesII of *C.reinhardtii* (CrMAnII), *A.thaliana* (AtManII), *D.melanogaster* (DmManII) and Human (HsManII) are in color. Conserved residues, which are involved in zinc ion binding and active site formation in the *D.melanogaster* enzyme, are marked at the top with an asterisk.

2.1.3. Identification of a β(1,2)-xylosyltransferase (CrXylT) ortholog in *C.reinhardtii.*

One of the important plant-specific glycosyltransferase activities is β(1,2)-xylosyltransferase (XylT), which is a medial-Golgi-localized type II membrane protein that is classified into glycosyltransferase family 61 of the *Carbohydrate Active Enzyme* database. XylT transfers a xylose residue from the donor substrate, UDP-xylose, to the core mannose of N-glycans via a β(1,2)-linkage, a reaction which is essential for plant-specific N-glycans (Bencur *et al.,* 2005) The *Xylt* gene has been identified in *Arabidopsis thaliana* (Strasser *et al.,* 2000), *Zea mays* (Bondili *et al.,* 2006), and *Medicago sativa* (Sourrouille *et al.,* 2008) and the enzymatic properties and subcellular localization signal of the *Arabidopsis* XylT protein have been determined (Pagny *et al.,* 2003).

It is demonstrated that the CT domain of XylT is dispensable for enzymatic activity (Bencur *et al.,* 2005). XylT has a proline rich domain (P), which has been suggested as a region required for XylT activity in *Arabidopsis* (Pagny *et al.,* 2003). However, most of these proline residues are not found in XylT sequence from other plant species (Léonard *et al.*, 2004). Interestingly, the proline-rich region of *A.thaliana* XylT is preceded by the tetrapeptide sequence KPWP. This KPWP motif is also present

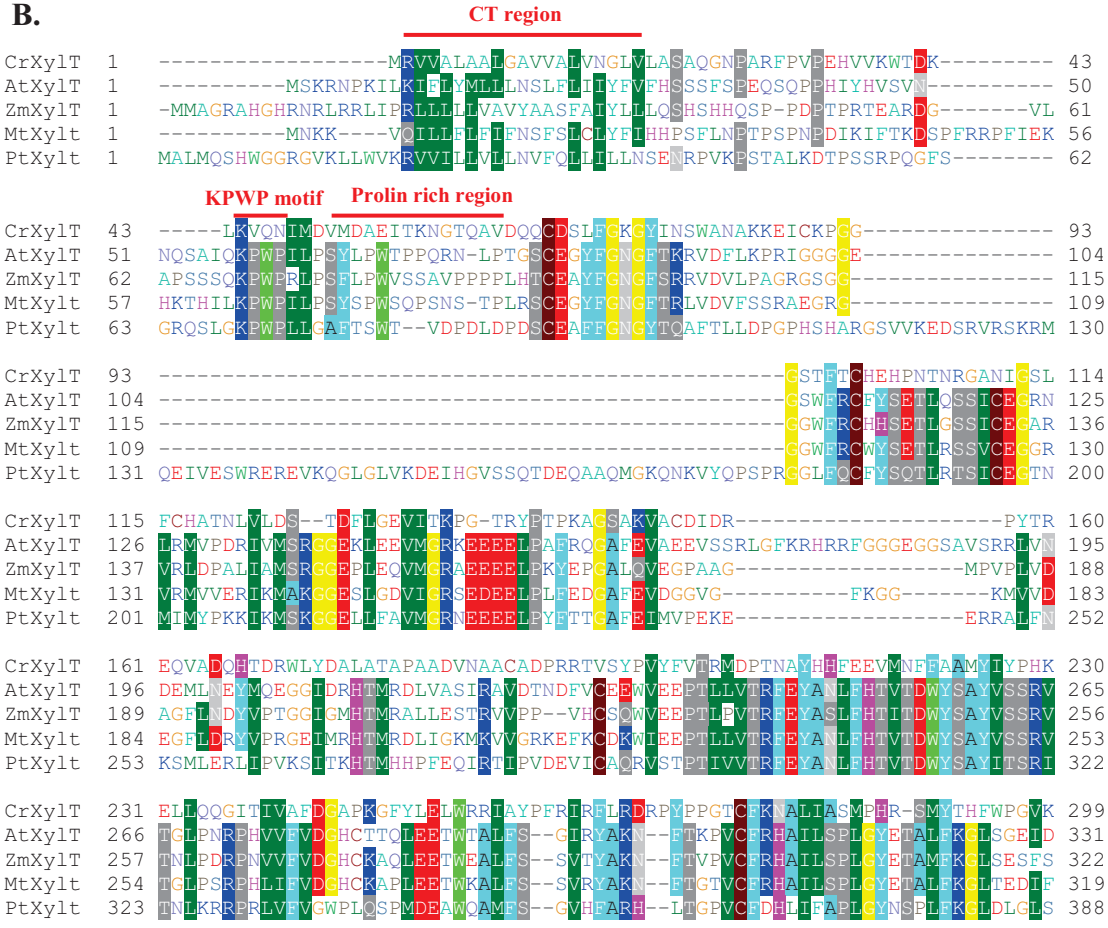
in other XylT sequences available at present (Léonar *et al.,* 2004) and this motif is essential for the generation of the active enzyme.

In silico analysis of *C.reinhardtii* genome revealed the presence of one potential gene encoding for a putative β(1,2)-xylosyltransferase (CrXylT). This potential gen presents 9 exons and 8 introns (Figure 2.3A) and the putative CrXylT exhibits 82% of identity with *Arabidospis thaliana*; 55% with *Zea May;* 60% of identity with *Medicago trunculata* and 61% of identity with *Physcomitrella patens* (Figure 2.3B). The sequence presents the appropriate type II membrane protein topology with a transmembrane domain between residues 3 to 24 in the N-terminus of the protein (VVALAALGAVVALVNGLVLASA) as was predicted using SOSUI server database (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html).

The putative CrXylT of *C.reinhardtii* does not present the proline-rich region and doesn´t have completely preserved the tetrapeptide sequence KPWW as *A.thaliana, D.melanogaster, M.trunculata* and *P.patens* (Figure 2.3B).

A.

B.



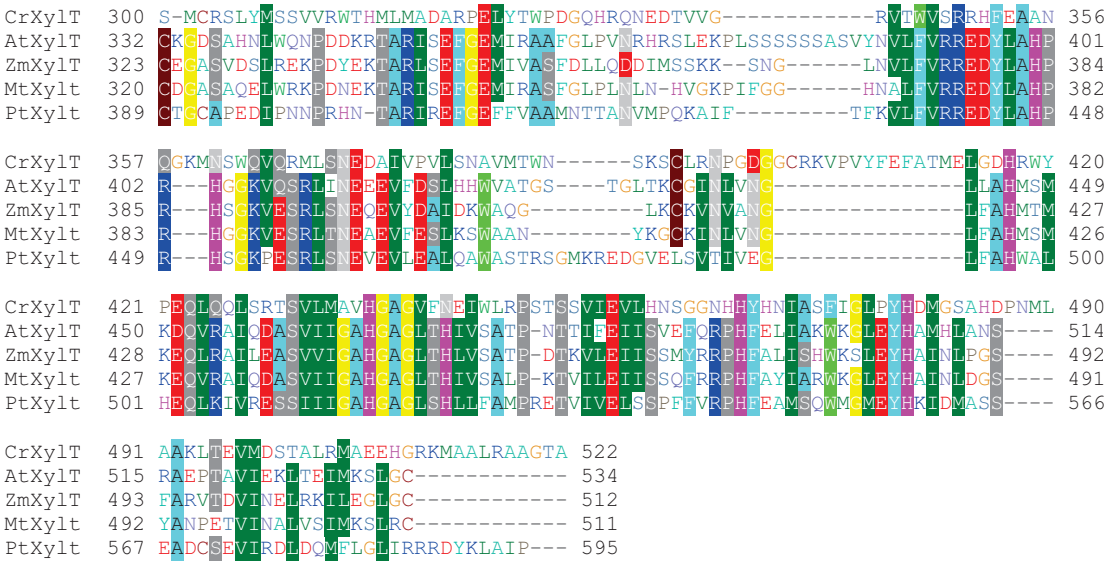


Figure 2.3: β(1,2)-xylosyltransferase of family GT61*. A. *Arrangement of the potential crxylt gene with 9 exons and 8 introns. Red boxes are exons and arrows indicate primers designed to PCR amplification. **B. *Aminoacid sequence alignment of the Chlamydomonas CrXylT***. Identical aminoacids of the xylosyltransferases in *C.reinhardtii* (CrXylT), *A.thaliana* (AtXylT), *Zea Mays* (ZmXylT), *Medicago trunculata* (MtXylT) and Phsycomitrella *patens* (PtXylT) are in color.

2.1.4. Identification of a α(1,3)-fucosyltransferase (CrFT1) ortholog in *C.reinhardtii.*

Other important type of plant-specific glycosyltransferases are α(1,3)-fucosyltransferases (FT1), which are Golgi-localized type II membrane proteins, classified into glycosyltransferase family 10 (GT10) of the *Carbohydrate Active Enzyme* database and PF00852 in Pfam database. FT1 transfer a fucose residue from the donor substrate, GDP-fucose, to a GlcNAc residue of the N-glycan core, via a α(1,3)-linkage, a reaction that is essential for plant-specific N-glycans.

α(1,3)fucosyltransferases contain three domains that are highly conserved in species from different kingdoms.

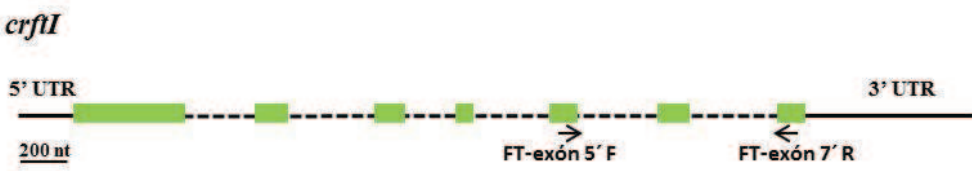
- Motif I that contains the amino acid sequence SSDV.
- Motif SNC that contains the amino acid sequence SNC (G/A) A (R/H) N.
- Motif II that contains the amino acid sequence YKFXXAFENXXXXDYITEKXXQ

In silico analysis of *C.reinhardtii* genome revealed the presence of a gene encoding for a putative α1,3-fucosyltransferase (CrFT1). This gene presents 7 exon and 6 introns (Figure 2.4A) and the putative CrFT1 protein exhibits 31% of identity with *Arabidospis thaliana* FT12; 34% of identity with *Arabidopsis thaliana* FT11 and 27% with *Droshopila melanogaster* (Figure 2.4B). When we checked to find the type II membrane protein topology typical of Golgi glycosyltransferases CrFT1 does not completely fulfill the requirements of hydrophobicity to safely predict a transmembrana

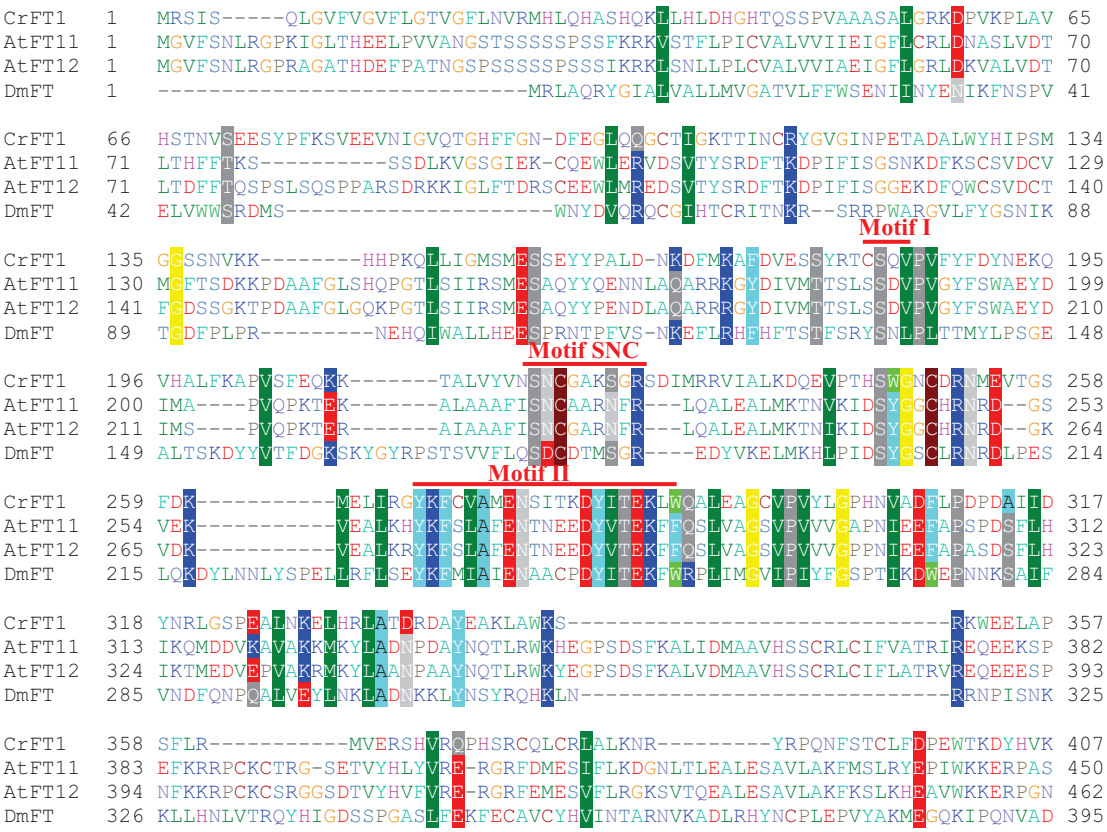
domain in its N-terminus using SOSUI server prediction. Nevertheless this is also the case for the already well characterized *D. melanogaster* FT1 (DmFT) (see Figure 2.4B).

This CrFT1 candidate exhibits the motif II and the motif SNC (G/A) A. This conserved motif II is present in animals, plants and insects (Oriol *et al.,* 1999; Wilson *et al*., 2001). Lysine residues (K) present in this motif, which is involved in recognition and catalysis of guanosine-diphosphate (GDP)-fucose, are conserved in *Chlamydomonas reinhardtii* (Figure 2.4B). The motif SNC (G/A) A is characteristic of α(1,3)-fucosyltransferases in plants. However, *Chlamydomonas* CrFT1 does not present the motif I (SSDV) characteristic of plants that have been proposed to be involved in coordinating a divalent metal ion, required for binding of sugar nucleotide donor. This motif is not present in α(1,3)-fucosyltransferases of animals, diatoms and insect as *D.melanogaster* (Figure 2.4B).

A.



B.



```
CrFT1   407 ------------------------------------------------- 407
AtFT11  451 LRGDGKLRVHGIYPIGLTQRQALYNFKFEGNSSLSTHIQRNPCPKFEVVFV 501
AtFT12  463 LKGDKELKIHRIYPLGLTQRQALYNFKFEGNSSLSSHIQNNPCAKFEVVFV 513
DmFT    396 WRAAMEVGQCQAKVLDEFFRRDIGFNDAEFDAELNRRIEGNNCSNSSNT-- 444
```

Figure 2.4: α(1,3)-fucosyltransferase of family GT10*. A. *Arrangement of the potential crft1 gen with 7 exons and 6 introns and the flanking regions 5' and 3' UTR. Green boxes are exons and arrows indicate the primers designed to PCR amplification. **B. *Aminoacid sequence alignment of Chlamydomonas CrFT1*.** Identical aminoacids of the α1,3-fucosyltransferases in C.reinhardtii (CrFT1), A.thaliana (AtFT11), A.thaliana (AtFT12) and D.melanogaster (DmFT1) are in color.

2.2. Molecular dissection of *Chlamydomonas* N-glycosilation pathway

Once we found the potential orthologous sequences of genes encoding for Golgi localized glycosyl hydrolases and glycosyltransferases in *C.reinhardtii* genome, primers were designed to amplify an internal region of each putative gene of *Chlamydomonas* pathway (See Materials and Methods, section 2.4.5 and Figure 2.1A, 2.2A, 2.3A and 2.4A).

We isolated genomic DNA and total RNA from cells of *Chlamydomonas* wild type cc503 strain cells growing under high CO_2 conditions. We have confirmed by PCR analysis the presence of these genes encoding for the putative glycosyl hydrolases and glycosyltransferases enzymes in *Chlamydomonas* genome (Figure 2.5A). The expression of all four genes in *Chlamydomonas* was analyzed by semi-quantitative RT-PCR analysis. Figure 2.5B shows that *C.reinhardtii* genes for α(1,2)-mannosidase I (c*rmanI*), Mannosidase II (c*rmanII*), α1,3-fucosyltransferase (c*rft1*) and β(1,2)-xylosyltransferase (c*rxylt*) are expressed in cells growing under high CO_2 conditions.

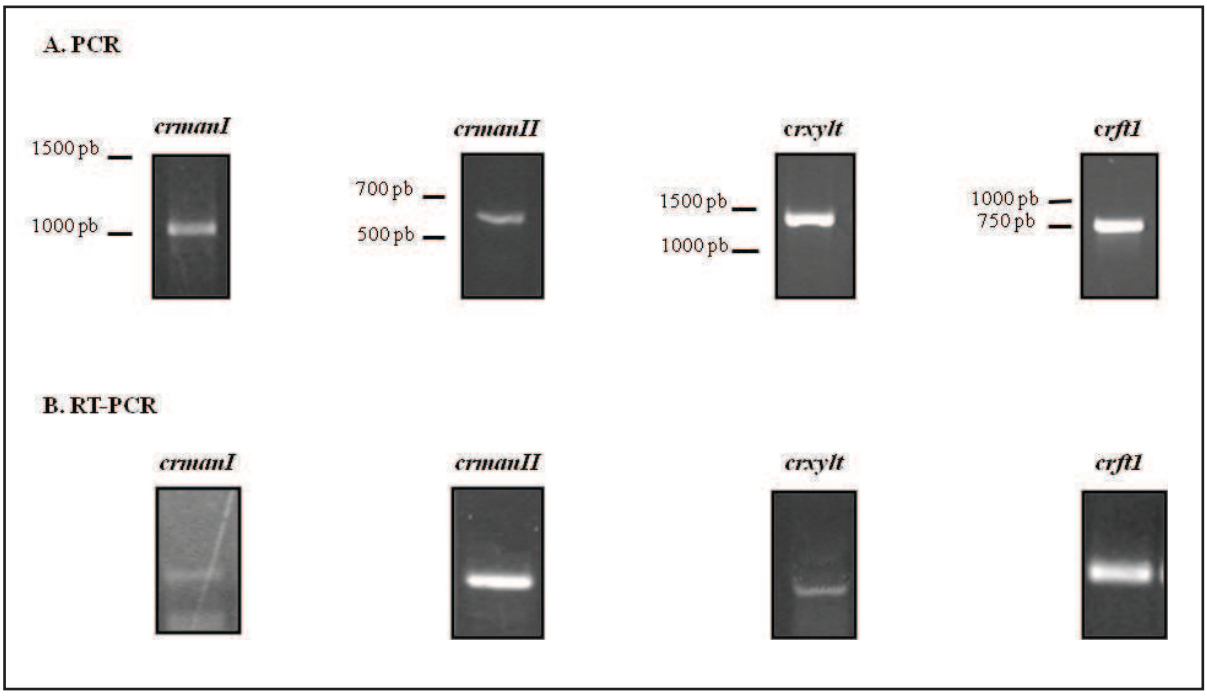


Figure 2.5: Presence and expression of crmanI, crmanII, crft1 and crxylt genes in C.reinhardtii wild type cc 503 strain. A: PCR analysis. B: Semi-quantitative RT-PCR analysis.

At the moment, we know that *C.reinhardtii* contains N-glycoproteins in the chloroplast and these N-glycoproteins are transported through the endomembrane system (ER and Golgi).

In addition, the *in silico* analysis of *Chlamydomonas* genome allowed us to identify orthologs for the enzymes involved in the biosynthesis of the N-glycans linked to glycoproteins (CrManI, CrManII, CrXylT and CrFT1). Surprisingly, *Chlamydomonas* genome does not present orthologs for GntI and GntII. However, it has been established that the action of GntI and GntII is a pre-requisite for the action of other glycosyltransferases and the formation of complex type N-glycans in plants and other algae (Johnson and Chrispeels, 1987; Tekuza et al., 1992; Strasser et al., 1999; Bakker et al., 1999).

All these results suggested that the N-glycosylation biosynthetic pathway in this green alga seems to be different and simpler than the one found in plants.

CHAPTER 3:

Characterization of fucosyltranferase orthologs identified in *Chlamydomonas reinhardtii.*

Other important aim of this work was the functional characterization of the key enzymes involved in the N-glycosylation biosynthetic pathway of *C. reinhardtii*. For this purpose we started with CrFT1, a putative α(1,3)-fucosyltransferase.

3.1 CrFT1, an α(1,3)-fucosyltransferase.

Many glycoproteins, glycolipids and oligosaccharides contain fucose, which is glycosidically linked to galactose, glucose, N-acetylglucosamine or directly to proteins. Fucosyltransferases are inverting enzymes that transfer fucosyl residues from GDP-β-L-fucose to Gal in an α(1,2)-linkage, to GlcNAc in α(1,3)-, α(1,4)-, or α(1,6)-linkages (Breton *et al.*, 1998). The majority of fucosyltransferases need divalent cations for their full activity (Staudacher, 1996). Since all fucosyltransferases utilize the same nucleotide sugar as substrate, their specificity will probably reside in the recognition of the acceptor in relation to the type of linkage formed (Breton *et al.*, 1998).

The big group of glycosyltransferases, to which fucosyltransferases belong, has been classified into different families based on the character of their activated donor substrate (usually a nucleotide-diphospho-sugar), the type of sugar which is transferred, and whether the enzyme forms a α- or β-glycosydic linkage.

In the Carbohydrate Active Enzyme (CAZy) database (http://www.cazy.org/, Coutinho and Henrissat, 1999), which is a wide database aimed at classifying enzymes which act on sugars, one can find more than 40000 glycosyltransferase-related sequences. They are divided into 94 families, from GT-1 to GT-94 (plus non-classified sequences) using sequence-based classification (Campbell *et al.*, 1997; Coutinho *et al.*, 2003; http://www.cazy.org/CAZY/

On the basis of protein sequence similarities in their catalytic domains, fucosyltransferases are mostly classified into 6 GT families (GT10, 11, 23, 37, 65, and 68) according to the CAZy database. However, the presence of three conserved peptide motifs shared by α(1,2)-fucosyltransferases, α(1,6)-fucosyltransferases, and protein-O-fucosyltransferases, suggests that they originated from a common ancestor (Breton *et al.*, 1998; Oriol *et al.*, 1999; Chazalet *et al.*, 2001; Martinez-Duncker *et al.*, 2003). Therefore, two superfamilies have been defined for fucosyltransferases; one containing the α(1,3)- and α(1,4)-fucosyltransferases that classify into GT10 family, and the other one which includes fucosyltransferase activities that classify into families 11, 23, 37, 65, and 68.

α(1,3)-fucosyltransferases transferring fucose to the inner GlcNAc in the core of N-glycans are called Core type, while α1,3-fucosyltransferases adding fucose to the GlcNAc residues in the branches of N-glycans or other glycoconjugates are called Lewis type (Chen *et al.*, 1998; Wilson, 2002).

As we explained in the Chapter 2, the FT1 GT10 family have the typical structure of type II transmembrane proteins consisting in: a short N-terminal cytoplasmic tail, a transmembrane domain, a stem region and a globular C-terminal catalytic domain containing the conserved peptide motifs (Martin *et al.*, 1997; Grabenhorst and Conradt, 1999; Holmes *et al.*, 2000; Breton *et al.*, 1998; Chazalet *et al.*, 2001).

3.1.1 Obtaining the 5′-UTR and 3′-UTR regions of the potencial gene coding for α(1,3)-fucosyltransferases of *Chlamydomonas reinhardtii.*

Once we got evidence for the presence and expression of a sequence coding for a α(1,3)-fucosyltransferase in *C. reinhardtii*, the next step was to identify the gene transcript, including the 5′-UTR and 3′-UTR regions. For this purpose we performed the amplification of 5′ and 3′ cDNA ends by RACE technique (see section 2.4.7 of Materials and Methods).

For that we started from total RNA isolated from *Chlamydomonas* wild type cells grown under high CO_2 conditions and we synthesized cDNA before performing 5′ and 3′ cDNA amplification by RACE techniques.

RACE products were cloned into pGEM T-Easy vector and we carried out transformation in *E.coli* DH-5α, plasmid DNA isolation and digestion with restriction enzyme *EcoRI* was done to select those colonies that contained the vector with the appropriate cloned fragment.

Plasmidic DNA of colonies that contained the vector with an appropriate fragment size were sequenced. The results of sequencing allowed us to precise 5′and 3′ends of α(1,3)-fucosyltransferase gene of *Chlamydomonas*, as well as initiation and stop codons for the translated protein (Figure 3.1).

TCATATCGCATTATGTAAAATAAACTGCAAAT**ATG**CGGAGCATAAGTCAACTTGGCGTGTTTGTGGGCGTCTTTCTCG
GGACAGTGGGCTTTCTTAATGTGCGAATGCACTTACAACACGCATCACACCAAAAGCTTCTTCACCTAGATCATGGGC
ACACGCAGTCGTCGCCCGTAGCTGCAGCCTCAGCGCTGGGGCGCAAGGACCCGGTTAAGCCGCTTGCTGTCCATTCAA
CAAACGTTAGCGAGGAGAGCTATCCCTTTAAGAGTGTGGAGGAGGTCAACATTGGAGTCCAAACCGGGCATTTCTTTG
GCAACGACTTCGAGGGGTTGCAGCAAGGCTGTACGATAGGGAAGACGACGATTAATTGCCGGTATGGGGTGGGCATCA
ACCCGGAGACAGCAGACGCGCTGTGGTACCACATCCCATCATGGAGTCGTCCGAGTACTACCCGGCCCTGGACAACAA
GGACTTCATGAAGGCCTTTGACGTGGAGTCCTCCTACCGCACCTGCTCCCAGGTGCCCGTCTTCTACTTTGACTACAA
CGAGAAGCAGGTGCACGCGCTGTTCAAGGCGCCGGTGTCGTTCGAGCAAAAGAAGACGGCGCTGGTGTACGTCAACAG
CAACTGCGGCGCCAAGAGCGGCCGCTCCGACATCATGCGCCGGGTGATTGCGCTCAAGGACCAGGAGGTGCCCACACA
CAGCTGGGGCAACTGCGACCGCAACATGGAGGTGACTGGCTCGTTCGACAAGATGGAGCTGATCCGCGGCTACAAGTT
CTGTGTGGCCATGGAGAACTCCATCACCAAGGACTACATCACCGAGAAGCTGTGGCAGGGCGGACTTCCTGCCCGACC
CCGACGCCATCATCGACTACAACAGGCTGGGCTCGCCGGAGGCGCTCAACAAGGAGCTGCACCGGCTGGCCACCGACC
GCGACGCCTACGAGGCCAAGCTGGCGTGGAAGTCGCGCAAGTGGGAGGAGCTGGCGCCCTCCTTCCTCAGGATGGTGG
AGCGCAGCCACGTGCGCCAGCCGCACAGCCGCTGCCAGCTGTGCCGCCTGGCGCTCAAGAACCGCTACCGGCCGCAGA
ACTTCAGCACCTGCCTGTTCGACCCCGAGTGGACCAAGGACTACCACGTCAAG**TAG**AGGGGAAGGGGTGAGGTGGCGG
GCGCCGCGGCGGCAGCAGCGGCGCAGTAGTGGTGATGGAGGGGTGGTAGAGAGGCATGGGTGACATTAGCAGCGAGCG
CTCCAGGCAGGCAGGAAGCTGTGACGTGAGCATAACGCGGCATTCCTCCACGTGTATGCCTATATGATTAAAAGTAAG
AGCTGTACTGCACGTGTCAACCGTATGAGAGCTGGTTTGGTACACTTGTATTTGCGCGAGCACTGCGGCATGCTGATG
ACTCACTGCGCCCGGGGTGGGTAGGGCCGGGTGGAGCATGTTCTACCAAGTGTGAACTGCAGTAGTAGCCTTGCCAGT
CCTGCACTGCCACCGGATATGTATTGCATGGTGTTCACGAAAACGCACATGATGCGCCATCGGGGTGGAGAGAAGTGC

TGCGGCATGCATTGTGTTGGCGAGTGCCGGTGGCCCGGTGCAAAAGTCGGCAACCCGGTGAACAGATGTGGCAGGTCG
TGGATCTGCTTGTAAAGGGCGAATGGCA

Figure 3.1: cDNA sequence of Chlamydomonas α(1,3)-fucosyltransferase gene with the 5´-UTR and 3´-UTR regions. Exons are marked in different colors and the 5´-UTR and 3´-UTR ends are colored in grey. The initiation and stop codons are underlined.

3.1.2. Obtaining the complete coding region of the gene *crft1.*

Once we identified the cDNA sequence of α(1,3)-fucosyltransferase of *Chlamydomonas,* we decided to obtain the complete coding region and use it as a tool for a different approach.

We performed a PCR amplification using the cDNA synthesized from total RNA isolated from *Chlamydomonas* wild type cells grown under high CO_2 conditions and using specific primers FT-ClonR and FT-ClonF (See Materials and Methods, section 2.4.6) we obtained a fragment of 1116 bp, the proper size of the expected cDNA (Figure 3.2**).**

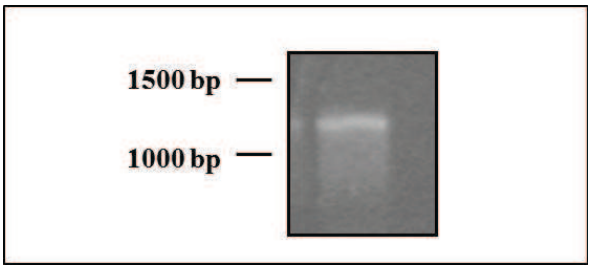


Figure 3.2: Gene cloning of crft1 cDNA from Chlamydomonas. cDNA was synthesized from total RNA isolated from *Chlamydomonas* wild type cells grown under high CO_2 conditions and a PCR amplification with specific primers FT-clonR y FT- clonF was performed. The PCR product was resolved on an agarose gel (1.5%).

PCR products were cloned into pGEM T-Easy vector and we carried out the transformation in *E.coli* DH-5α, the plasmid DNA isolation and digestion with restriction enzyme *EcoR I* was done to select those colonies that contained the vector with the cloned fragment that were sequenced to be sure that we cloned the *crft1*gene coding region (Figure 3.2).

3.1.3 Expression of α(1,3)-fucosyltransferase of *Chlamydomonas* in response to a change in growing conditions.

To know if *crft1* expression is regulated by environmental conditions, total RNA was isolated from *Chlamydomonas* wild type in high CO_2 (0h) and acclimated to low CO_2 (2h, 4h y 6h). cDNA was synthesized from total RNA isolated and PCR reactions were performed with primers FT-exon5´F and FT-exon7´R (See Materials and Methods, section 2.4.6), to analyze expression *of Chlamydomonas* α(1,3)-fucosyltransferase gene (*crft1*) (Figure 3.3A).

Transcript levels of the constitutive gene β2-tubulin (Figure 3.3B) were used as control of sample loading, using β2TUBF and β2TUBR primers (See Materials and Methods, section 2.4.6).

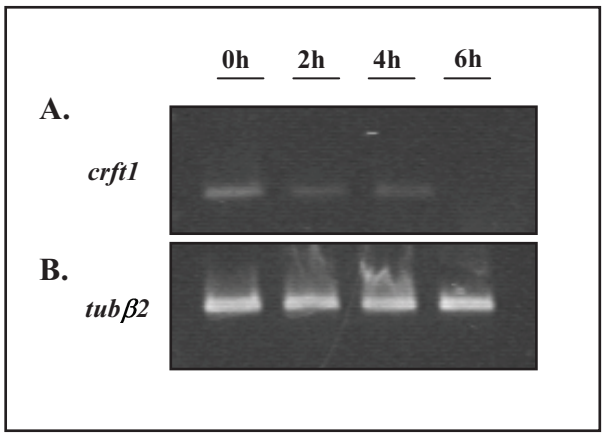


Figure 3.3: Semi-quantitative RT-PCR amplification of RNA extracted from cells growing under high-CO_2 (0h) or acclimated to low-CO_2 conditions for 2h, 4h and 6h. RT-PCR with specific primers β2TUBR and β2TUB; RT-FT R and RT-FT F were performed. The PCR products were resolved on agarose gels (1.5%). **A**. α(1,3)- fucosyltransferase **B.** β-Tubulin.

Semi-quantitative RT-PCR analysis show that expression of α(1,3)-fucosyltransferase (*crft1*) gene decreased gradually over time of acclimation to low CO_2 being completely down-regulated within the first 6h after transferring cells from high- to low-CO_2 conditions.

If the enzyme encoded by *Chlamydomonas crft1* gene is responsible for the fucosylation of proteins, the repression of the expression of this gene during acclimation to low CO_2 must be reflected in a gradual decrease of protein fucosylation levels. To check this hypothesis, total extract (TE), chloroplasts (CL) and stromal (ST) fractions were isolated from *Chlamydomonas* wild type cells growing under high-CO2 (0h) or acclimated to low-CO_2 conditions for 6 h and analyzed using specific antibodies against α(1,3)-fucose residues (Figure 3.4A) and synthesis *de novo* of the mitochondrial CA protein was used as a marker of both mitochondrial contamination and induction of CCM (Figure 3.4B).

Figure 3.4A shows that in total extract (TE), chloroplast (CL) and stromal (ST) fractions of cells acclimated to low CO_2 conditions during 6 h, fucosylation levels of endogenous proteins of *Chlamydomonas reinhardtii* are decreased comparing to the levels observed in the samples obtained from cells grown in high CO_2, so the decline in gene expression is correlated with a decrease in the α(1,3)-fucosylation level of endogenous *Chlamydomonas* glycoproteins.

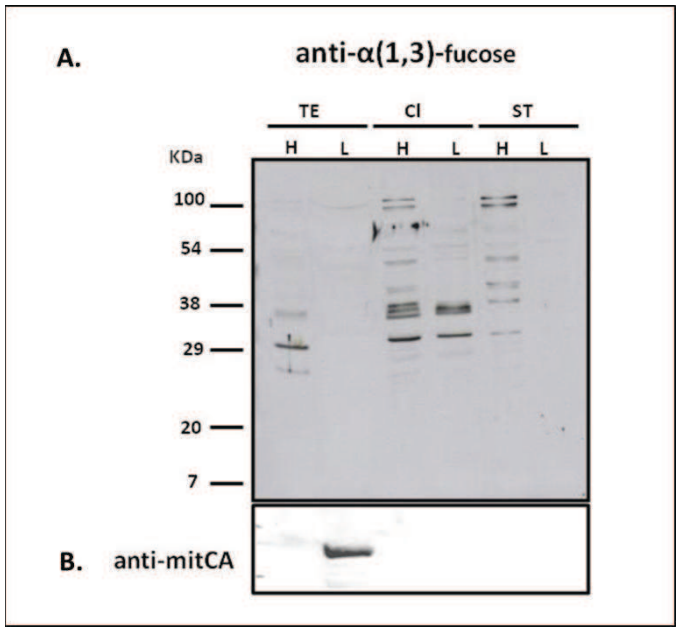


Figure 3.4: Fucosylation levels of endogenous proteins in Chlamydomonas. A. 1 µg of chlorophyll of total cell extracts, chloroplast and stroma were separated by electrophoresis on 10% acrylamide gels and transference onto nitrocellulose membranes was made**.** Immunodetection was performed with a primary antibody raised against α(1,3)-fucose residues and a secondary antibody coupled to horseradish peroxidase. **B.** Immunodetection was performed with a primary antibody raised against mitochondrial CA protein and a secondary antibody coupled to horseradish peroxidase. TE: Total extract; CL: Chloroplasts; ST: Stroma; H: Cells grown in high CO_2; L: Cells grown in low CO_2 for 6h.

To check whether the fucosylation pattern of protein is recovered over time, total extract, chloroplast and stroma from *Chlamydomonas* wild type cells were isolated after 24 h acclimation to low CO_2 conditions and were compared with fucosylation levels of proteins from total extract, chloroplast and stroma from *Chlamydomonas* wild type cells grown in high CO_2 conditions. As the previous assay, samples were analyzed using specific antibodies against α(1,3)-fucose residues (Figure 3.5). Figure 3.5 shows that fucosylation levels of endogenous proteins from cells grown under low CO_2 conditions are similar to those coming from cells grown in high CO_2 conditions. This result clearly indicates that the fucosylation level of endogenous proteins was recovered after 24 h acclimation to low CO_2 conditions and that α(1,3)-fucosyltransferase expression seems to be recovered after 24h of acclimation to low CO_2 conditions.

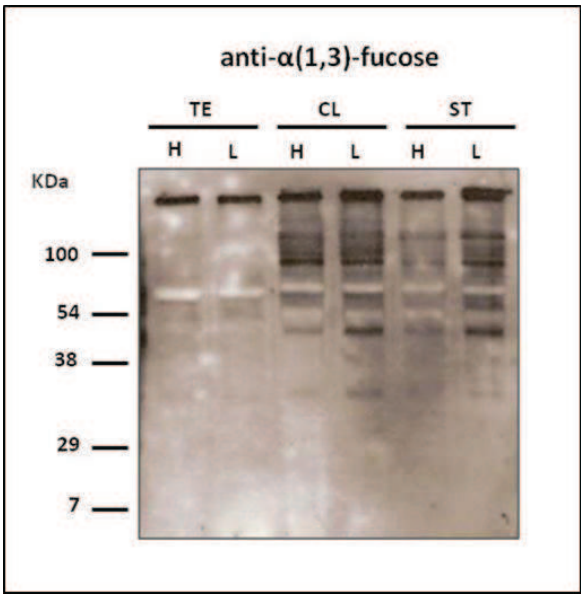


Figure 3.5: Fucosylation levels of endogenous proteins in Chlamydomonas. 1 µg of chlorophyll of total cell extracts, chloroplast and stroma were separated by electrophoresis on 10% acrylamide gels and transference onto nitrocellulose membranes was made**.** Immunodetection was performed with a primary antibody raised against α(1,3)-fucose residues and a secondary antibody coupled to horseradish peroxidase. TE: Total extract; CL: Chloroplasts; ST: Stroma; H: Cells grown in high CO_2; L: Cells grown in low CO_2 for 24h.

3.1.4 Analysis of *Chlamydomonas* N-glycan sensitivity to commercial glysosidases. Localization of fucose residues.

As it has been mentioned above (see Chapter 2 of Results) we couldn´t find any orthologous of *gnt I* or *gnt II* genes in *Chlamydomonas*. However, studies on the glycan acceptor specificity of the α(1,3)-fucosyltransferase and β(1,2)-xylosyltransferase in plants show that the presence of a terminal residue of N-acetylglucosamine is a prerequisite for the transfer of α(1,3)-fucose and β(1,2)-xylose residues in N-glycan formation (Johnson and Chrispeels, 1987; Tekuza *et al.,* 1992; Lerouge *et al.,* 1998), indicating that the action of Gnt I and Gnt II is needed for adding α(1,3)-fucose and β(1,2)-xylose residues. The absence of Gnt I and Gnt II in *Chlamydomonas* genome raises the question about how and where is the fucose residue added in *Chlamydomonas*.

To answer these questions, we analyzed the sensitivity of *Chlamydomonas* N-glycans to two commercial glycosidases, Endo H and PNGase F. As it is explained in Materials and Methods (section 2.3.3), Endo H is an enzyme that releases high-mannose-type (not complex-type) glycosidic moieties from glycoproteins, hydrolyzing the O-glycosidic bond between the two molecules of N-acetylglucosamine of the N-glycan core (See Materials and Methods, section 2.3.3) generating a truncated sugar molecule with one N-acetylglucosamine residue remaining on the asparagine residue of the polypeptide chain. On the other hand PNGase F hydrolyzes the N-glycosidic bond between N-acetylglucosamine and asparagine of the polypeptide chain, but only if the

N-acetylglucosamine does not bear an α(1,3)- linked fucose residue as it is shown in Figure 3.6.

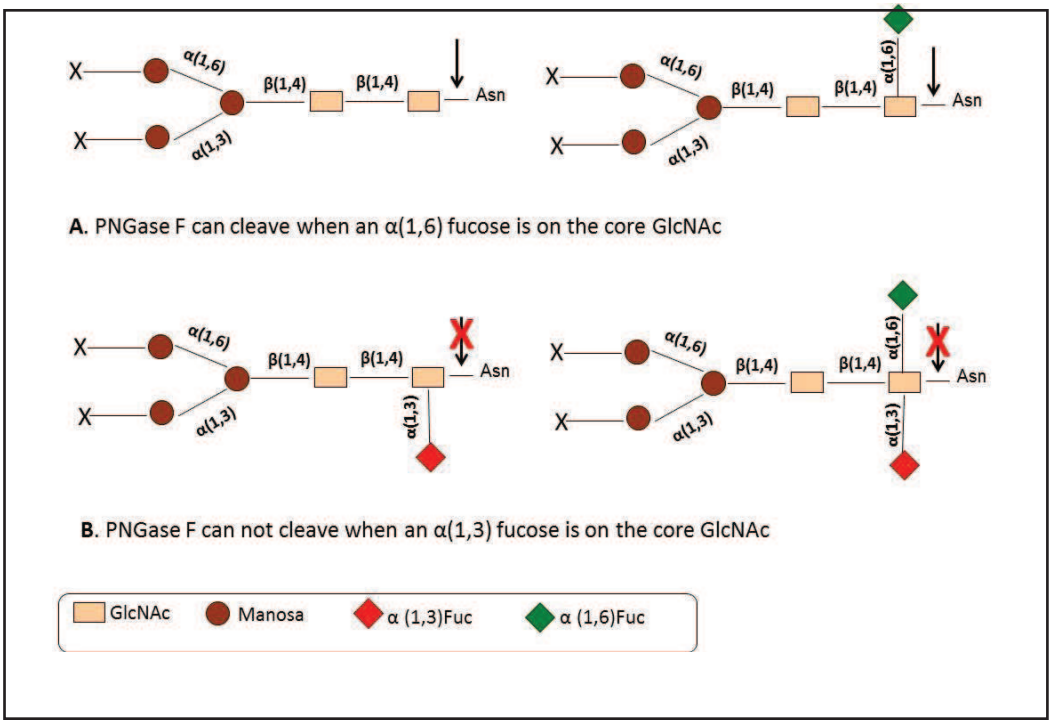


Figure 3.6: Scheme of PNGaseF cleavage mechanism. PNGase F is not able to cleave N-linked glycans from glycoproteins when the innermost GlcNAc residue is linked to alpha1-3. This modification is most commonly found in plant and some insect glycoproteins. Tretter. *et al*., (1991). Figure adapted from www.neb.com.

Total cell extracts (TE) and stromal fractions (ST) isolated from *Chlamydomonas* wild type cells grown in high CO_2 conditions were treated for 24 h with Endo H and PNGase F enzymes. After digestion, the treated samples were separated by acrylamide gel electrophoresis followed by a transference to nitrocellulose membranes to perform an Affinoblot assay with Con A lectine (Figure 3.7A) and immunodetection with antibody against α(1,3) fucose residues (Figure 3.7B).

As it is shown in Figure 3.7A and B, the pattern of N-glycans anchored to glycoproteins is the same for control and Endo H treated samples, and either after Con A detection or anti-α1,3-fucose detection, therefore indicating that *Chlamydomonas* N-glycans are not sensitive to Endo H cleavage, independently of their location in the chloroplast or not. As it occurs in plants, the lack of sensitivity to Endo H of N-glycans in *Chlamydomonas* indicates that they are complex-type N-glycans; even those recognized by Con A lectine, since they did not change the pattern after Endo H treatment (Figure 3.7A).

However, Figure 3.7A and B also shows that, against what occurs in plants, N-glycans anchored to glycoproteins in *Chlamydomonas* and detected with both, Con A and with antibodies against α(1,3)-fucose, are almost entirely digested by PNGase F.

The latter indicates that α(1,3)-fucose residues might not be linked to the proximal GlcNAc of N-glycan core, since otherwise they would block PNGase F action.

Therefore, these results pointed to the fact that CrFT1 does not add α(1,3)-fucose residues to the proximal GlcNAc of the quitobiose core, as plant FTases do. However, the algal *N*-glycans are not sensitive to Endo H digestion (H) indicating that *Chlamydomonas* endogenous N-glycoproteins might contain some type of residue that blocks the action of this glycosidase and are not just a high-mannose type N-glycan but an algal specific complex-type N-glycan**.**

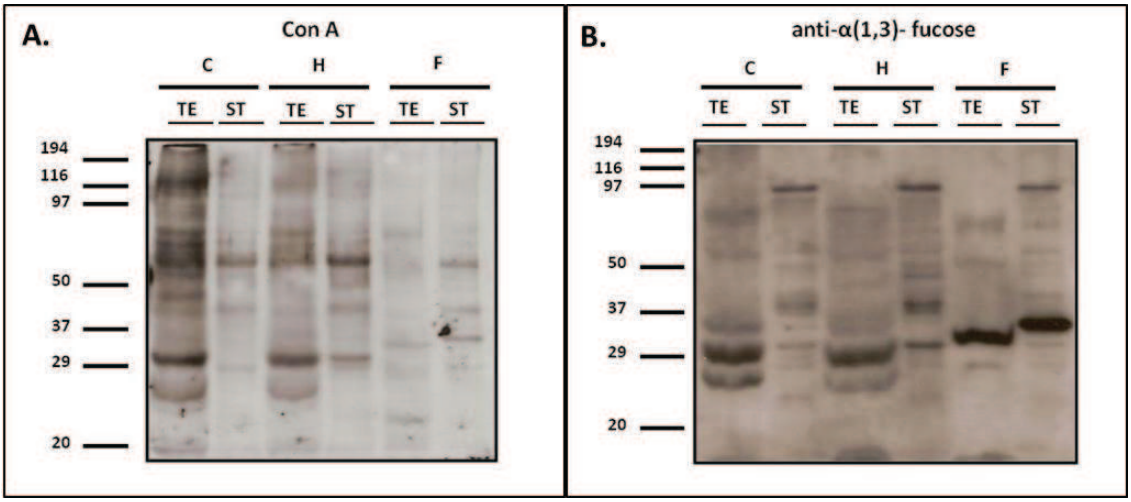


Figure 3.7: Sensitivity of the N-glycans to endoglycosidases Endo H and PNGase F. Total cell extracts (TE) and stromal fractions (ST) from *Chlamydomonas* wild type cells grown in high CO_2 conditions. Samples were digested with Endo-H and PNGase F for 24h and were separated by electrophoresis on 10% acrylamide gels and transference onto nitrocellulose membranes was made**. A.** Affinoblot based on the affinity of the N-glycans to Con A**. B.** 2 µg of chlorophyll were separated by electrophoresis on 10% acrylamide gels Immunodetection was performed with a primary antibody raised against α(1,3)-fucose residues and a secondary antibody coupled to horseradish peroxidase. TE: Total Extract; ST: Stromal; C: Control; H: Endo-H; F: PNGase F.

3.1.5 Analysis of N-glycans acceptor specificity of the *Chlamydomonas* CrFT1.

The possible addition of α(1,3)-fucose residue at a different position of that of the proximal N-acetylglucosamine as well as its action independent of GnT I, suggested that the glycan acceptor specificity of CrFT1 was different of α(1,3)-fucosyltransferases in plants. To confirm this hypothesis, we decided to express the coding region of *crft1* gene in a plant heterologous system and to analyze the requirements for its action.

As heterologous system we decided to use transient expression in protoplast of mesophyll cells from different lines of *Arabidopsis thaliana*: wild type Columbia 0+, a mutant defective in *gnt I* gene (*cgl1*) that exclusively presents high-mannose type N-glycans, therefore confirming the requirement of GnT I to synthesize complex-type N-glycans and a double mutant in the two α(1,3)-fucosyltransferases (*ft11/ft12*) present in *Arabidopsis* genome.

The above mentioned protoplasts were transfected with the construction represented in Figure 3.8A, including *crft1* coding region tagged with an HA epitope (CrFT1-HA) in the C-terminus and inserted in the polylinker region of pPE1000 plasmid (see Materials and Methods section 2.4.12). As negative control, the protoplasts from the three lines were transfected with water. Twenty four hours after transfection proteins were extracted from all experimental conditions and analyzed.

The first step was to check whether CrFT1-HA transgene was expressed in *Arabidopsis* protoplasts. Immunobloting analysis with an antibody raised against HA was performed. As we can see in figure 3.8C, HA epitope is only detected in protoplasts transfected with the transgenic plasmid in the three *Arabidopsis* lines, but not in protoplasts transfected with water.

Once we confirmed the expression of the HA tag in the three lines of protoplasts, we analyze the α(1,3)-fucosyltransferase activity of *Chlamydomonas* enzyme in the *Arabidopsis* system by analyzing the levels of protein fucosylation in the different protoplasts. We performed an immunobloting analysis with the antibody raised against α(1,3)-fucose residues (Figure 3.8B). Protein extracts from wild type protoplasts showed a very strong signal, typical of wild type plants, both in water and CrFT1-HA transfected extracts (Figure 3.8B, line 1 and 4). As expected, negative control extracts of protoplast obtained from both mutants, *ft11/ft12* and *cgl1*, had a residual labeling with α(1,3)-fucose antibodies (Figure 3.8B). However, those transfected with CrFT1-HA partially recovered fucosylation levels, especially in protoplast obtained from *cgl1*.

Data obtained from *ft11/ft12* transfected protoplast suggested that CrFT1 is able to add α(1,3)-fucose in complex type N-glycans lacking this residue. Nevertheless it seems to be more efficient adding fucose residues in high-mannose-type N-glycans lacking N-acetylglucosamine at the distal end, suggesting that, in some way, the glycan acceptor specificity is different. These results indicated that CrFT1 do not required N-acetylglucosamine in terminal position of the N-glycans to carry out its function, confirming that *Chlamydomonas* CrFT1 function is independent of GnT I.

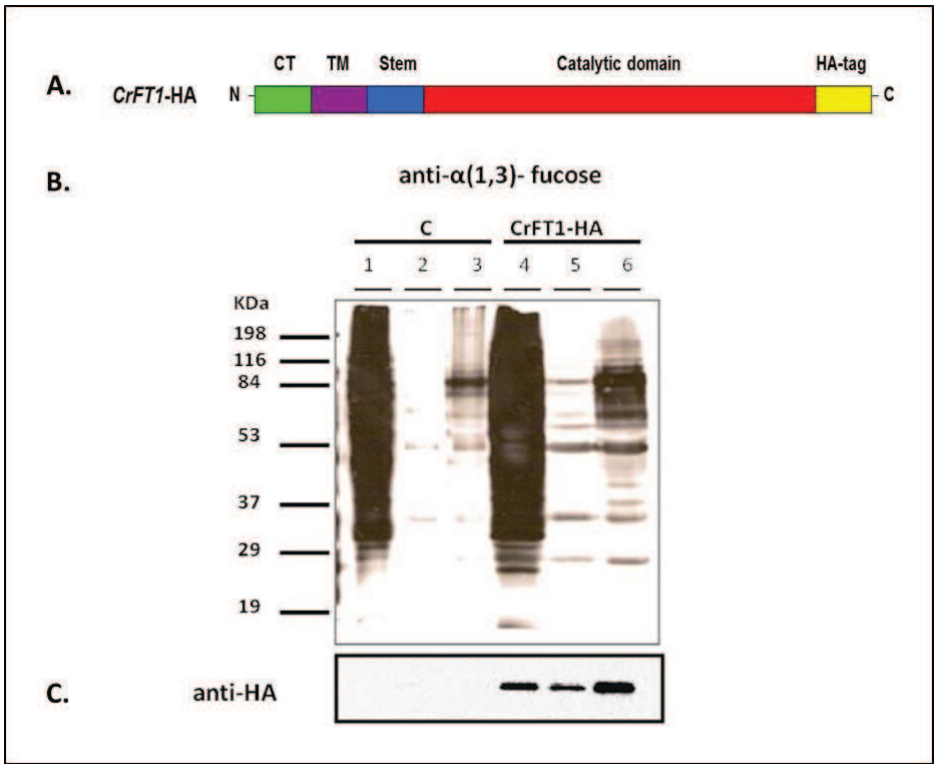


Figure 3.8: Endogenous level of fucosylation in transfected Arabidopsis protoplast with CrFT1 of Chlamydomonas. A. Construction of α(1,3)-fucosyltransferase coding region tagged in C-terminus with an HA epitope (CrFT1-HA) ***B.*** *A. thaliana* protoplasts were isolated and transfected with the fucosyltransferase of *Chlamydomonas* tagged with HA epitope on the C-terminus. 1 µg of chlorophyll samples were separated by electrophoresis on 10 % acrylamide gels and transference onto nitrocellulose membranes was made. Immunodetection was performed with primary antibody raised against α(1,3)-fucose residues and a secondary antibody coupled to horseradish peroxidase. **C**. Immunodetection was performed with anti-HA antibody and a secondary antibody coupled to horseradish peroxidase. C: protoplasts transfected with water; CrFT1-HA: protoplasts transfected with CrFT1-HA; 1 and 4: wild strain of *Arabidopsis;* 2 and 5: *FT11/12 Arabidopsis* mutant; 3 and 6: *cgl1 Arabidopsis* mutants.

3.1.6. Silencing of *crft1* gene by artificial microRNAs in *Chlamydomonas.*

Recently, different groups have demonstrated that the unicellular alga *Chlamydomonas reinhardtii*, like other multicellular organisms, contains miRNAs. These RNAs resemble the miRNAs of land plants in that they direct site-specific cleavage to target mRNA with miRNA-complementary motifs and, presumably, act as regulatory molecules in growth and development. Based on these finding, a novel artificial miRNA (amiRNA) system based on ligation of dsDNA oligonucleotides that can be used for specific high-throughput gene silencing in green algae have been recently developed by two different groups (Molnar *et al.,* 2007; 2009 and Zhao *et al.,* 2007; 2009).

As we explained in Materials and Methods (See section 2.4.14) we used an artificial microRNA construction (amiRNA) for silencing *crft1* gene (Figure 3.9). The construct was introduced in pChlamiRNA2 plasmid, following the method described by Molnar *et al.* (2009) (Figure 3.9). Nuclear transformation of *Chlamydomonas* cells was performed with the glass beads method (Kindle, 1980).

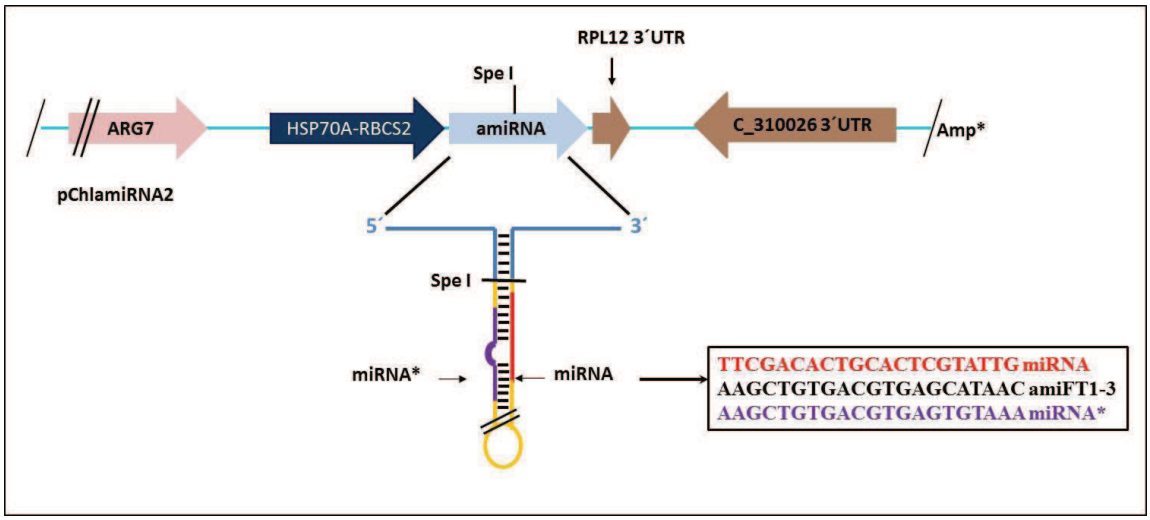


Figure 3.9: ***Schematic diagram of the amiRNA vector for crft1 gene silencing.***

We performed a preliminary screening of transgenic cell lines containing the amiRNA constructs. Semi-quantitative RT-PCR analyses showed that transformation with the amiRNA construct led to a decrease in the level of the *crft1* transcript in some of the transgenic cell lines when compared to the wild-type (cc425 arg2 cw92 strain) (Figure 3.10). Transcript levels of the constitutive gene β2-tubulin were measured to control the sample loading using β2TUBF and β2TUBR primers (Figure 3.10) (See Materials and Methods, section 2.4.6).

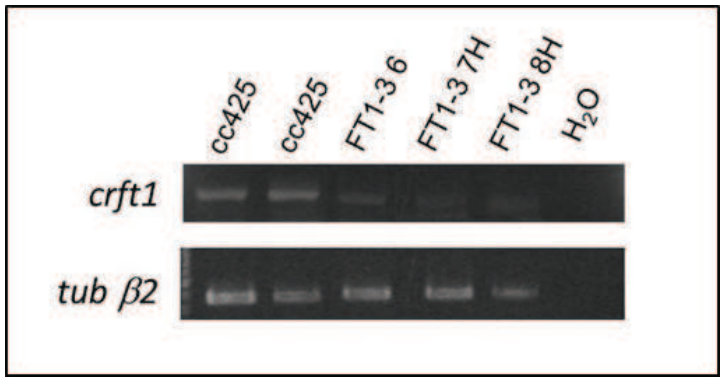


Figure 3.10: Semi-quantitative RT-PCR amplification of RNA extracted from cells growing under high-CO_2. PCR were performed with specific primers β2TUBR and β2TUB; and RT-FT R y RT-FT F. The PCR products were resolved on 1.5% agarose gels.

To confirm *crft1* silencing in the transgenic lines, we performed an immunoblot analysis of total cell protein extracts (10 µg of protein) from cc425 strain and some of the transgenic lines (denoted as FT1-3 6, FT1-3 7H and FT1-3 8H) with antibodies raised against α(1,3)-fucose residues. Figure 3.11A shows that the lower expression of *crft1* is correlated with a decrease in the level of fucosylation of the endogenous algal N-glycoproteins when compared to cc425 levels. As loading control, immunodetection was performed with antibodies against RuBisCo (Figure 3.11B).

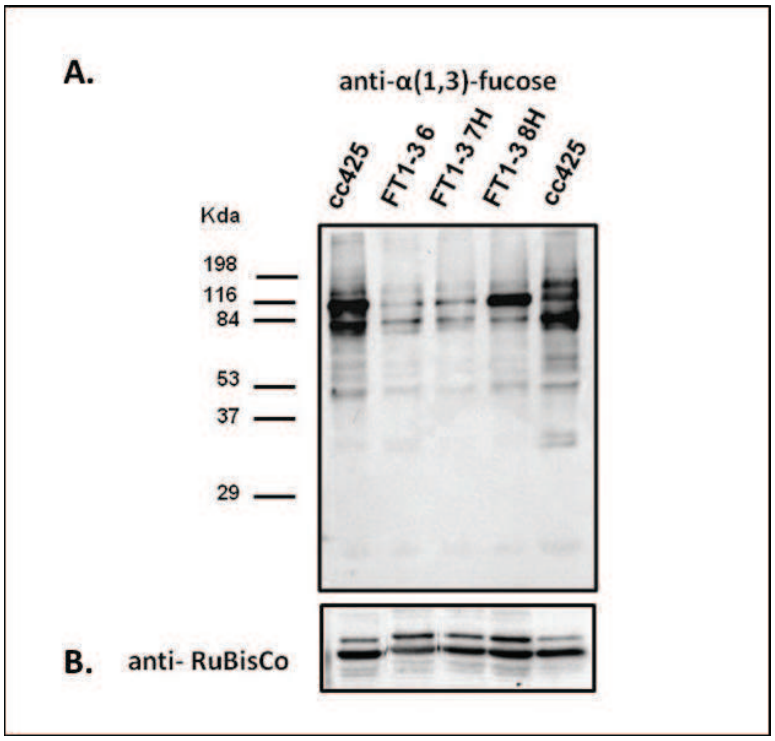


Figure 3.11: Endogenous levels of fucosylation of the proteins of transgenic lines of C.reinhardtii. 10 µg of protein of total cell extracts were separated by electrophoresis on 12% acrylamide gels and transference onto nitrocellulose membranes was made**. A.** Immunodetection was performed with a primary antibody raised against α(1,3)-fucose residues and a secondary antibody coupled to horseradish peroxidase. **B.** Immunodetection was performed with a primary antibody raised against anti-RuBisCo and a secondary antibody coupled to horseradish peroxidase. cc425: wild-type arg2 cw92 strain; FT1-3 6, FT1-3 7H and FT1-38H: different transgenic lines.

In order to check whether or not *crft1* silencing could affect the activity of the other enzymes involved in N-glycan biosynthesis, we performed immunoblot analysis of the transgenic lines and cc425 with antibodies raised against β(1,2)-xylose residues as well as affinoblot analysis against Con A lectine (Figure 3.12). The results showed that the transgenic lines presented β(1,2)-xylose residues and were recognized by Con A lectine as the same level as cc425 strain of *Chlamydomonas* used as recipient for the silencing strategy. Therefore silencing of *crft1* in these transgenic lines does not affect the function of the other enzymes of the N-glycosylation pathway in *Chlamydomonas.*

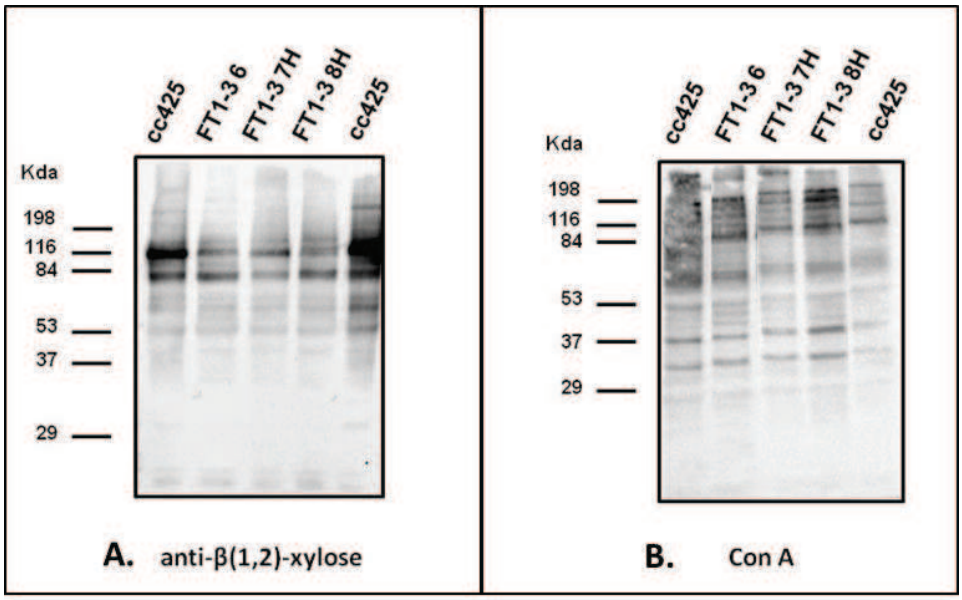


Figure 3.12: Glycosylation levels of the proteins of transgenic lines of C.reinhardtii. ***A.*** 10 µg of protein of total cell extracts were separated by electrophoresis on 12% acrylamide gels and transference onto nitrocellulose membranes was made**.** Immunodetection was performed with a primary antibody raised against β(1,2)-xylose residues and a secondary antibody coupled to horseradish peroxidase. **B.** 2 µg of–protein of total cell extracts were separated by electrophoresis on 12% acrylamide gels and transference onto nitrocellulose membranes was made**.** Affinoblot based on the affinity of the N-glycans to Con A lectine was made. cc425: wild-type arg2 cw92 strain; FT1-3 6, FT1-3 7H and FT1-38H: different transgenic lines.

3.2 CrFT8: The discovery of a new α(1,6)-fucosyltranferase in *C.reinhardtii*

Once we found that *Chlamydomonas* N-glycans were resistant to Endo H but not to PNGase F cleavage, we reasoned that there should be a residue linked to the proximal GlcNAc of the N-glycan core, which had to be compatible with PNGase F action but should interfere with Endo H action. Such a residue might be a α(1,6)-fucose, which is present in complex N-glycans of mammals and insects and does not interfere with PNGase cleavage.

In silico analysis of *C.reinhardtii* genome revealed the presence of one gene encoding for a putative α(1,6)-fucosyltransferase (CrFT8). This gene presents 8 exons and 7 introns (Figure 3.13A).

α(1,6)-fucosyltransferases catalyze the transfer of a fucose residue from GDP-fucose to the reducing terminal N-acetylglucosamine (GlcNAc) of Asn-linked oligosaccharides (N-glycans) via an α(1,6)-linkage, and is involved in biosynthesis of complex type N-linked oligosaccharides in glycoproteins of different organisms (Longmore and Schachter, 1982; Kobata, 1992; Miyoshi *et al.,* 1999). The reaction does not require any divalent cations or cofactors.

α(1,6)-fucosyltransferases belong to the GT23 family of glycosyltransferases in CAZy classification. These family of enzymes are typical type II membrane proteins, localized in the Golgi apparatus. Nevertheless, the putative CrFT8 protein doesn´t seem

to exhibit the appropriate transmembrane domain according to the SOSUI server prediction as well as FT8 protein from *Ciona intestinalis* (Figure 3.13B).

α(1,6)-fucosyltransferases from porcine brain and a human gastric cancer cell line have been purified and the cDNA cloned (Uozumi *et al.,* 1996, Yanagidani *et al*., 1997).

α(1,6)-fucosyltransferases contain three short regions that are highly conserved in all α(1,6)- (as human FUT8 or bacterial α(1,6)), but also in α(1,2)- and protein O-fucosyltransferases (Oriol *et al.,* 1999; Takahashi *et al.,* 2000; Chazalet *et al*., 2001; Martinez-Duncker *et al*., 2003).

The putative α(1,6)-fucosyltransferase (CrFT8) of *Chlamydomonas* exhibits 26% of identity with *Homo sapiens;* 23% of identity with *Droshopila melanogaster;* 29% of identity with *Caenorhabditis elegans* and 25% of identity with *Ciona intestinalis* (Figure 2.13B). The putative CrFT8 presents the Motif I, conserved in insects and other organisms, which is essential for activity, as well as the conserved motifs II and III (Figure 3.13B).

Human FUT8 contains eight cysteine residues (Cys-204, 212,218, 222, 230, 266, 465, and 472 (Figure 3.13B) (Yanagidani *et al.,* 1997) and have been identified all combinations of cysteines that form four disulfide bonds. Five cysteine residues (Cys-204, 212, 218, 222 and 230) of Human FUT8 are perfectly conserved among vertebrates, insect, nematodes and ascidian. Some of the four disulfide bonds of FUT8 which are strongly conserved may play important roles in the correct folding of the protein and /or its stability rather than for the enzymatic function of catalysis (Ihara *et al.,* 2007). The putative CrFT8 present only two conserved cysteine residues, but present other six cysteines in different position (Figure 3.13B).

A.

B.

```
CrFT8    1   ------------------------------MIAPGAYVGNRD------------DREEDGRQLYNSALAQRFIYRHQN--  36
HsFT8    1   ---MRPWTGS-----WRWIMLILFAWGTLLFYIGGHLVRDND-----HPDHSSRELSKILAKLERLKQQNEDLRRMAESL  67
DmFT8    1   MLLVRQLFGASANSWARALIIFVLAWIGLVYVFVVKLINTQGQQAAGESELNARRISQALQMLEHTRQRNEELKQLIDEL  80
CiFT8    1   -MRLRQFLHMDLR---WRTFIVTFSIACCTFFYMFQLTSRKS------RVIVEGKFTKTERWFHNNDAGESFDLQERQHI  70
CeFT8    1   ---MLKCIAA----------VGTVVWMTMFLFLYSQLSNNQS------GGDSIRAWRQTKEAIDKLQEQNEDLKSILEKE  61

CrFT8   36   -----PADCRSAKFLVAEFDAEGACPSRVG-------------------------------------------------- 61
HsFT8   68   RIPEGPIDQGPAIGRVRVLEEQLVKAKEQ----------------------IENYKKQTRNGLGK------------DHEI 114
DmFT8   81   MS--DQLDKQSAMKLVQRLENDALNPKLAPEVAGPEPESMFESAPADLRGWNNVAEGAPNDLEAGVPDHGEFEPSLEYEF 158
CiFT8   71   RNAMSDVEQIQAMVRHQLIKIYQESPKAQP---------------------------------------------------- 100
CeFT8   62   RQ-ERNDQHKKIMEQSHQLPPNPENPSLPK--------------------PEPVKEIISKPSILG---------PVQQEV 111

CrFT8   61   ------------------------------------IGSTIHMMSYGLTRAMMMGRVYIEE----------DGGQIWT  93
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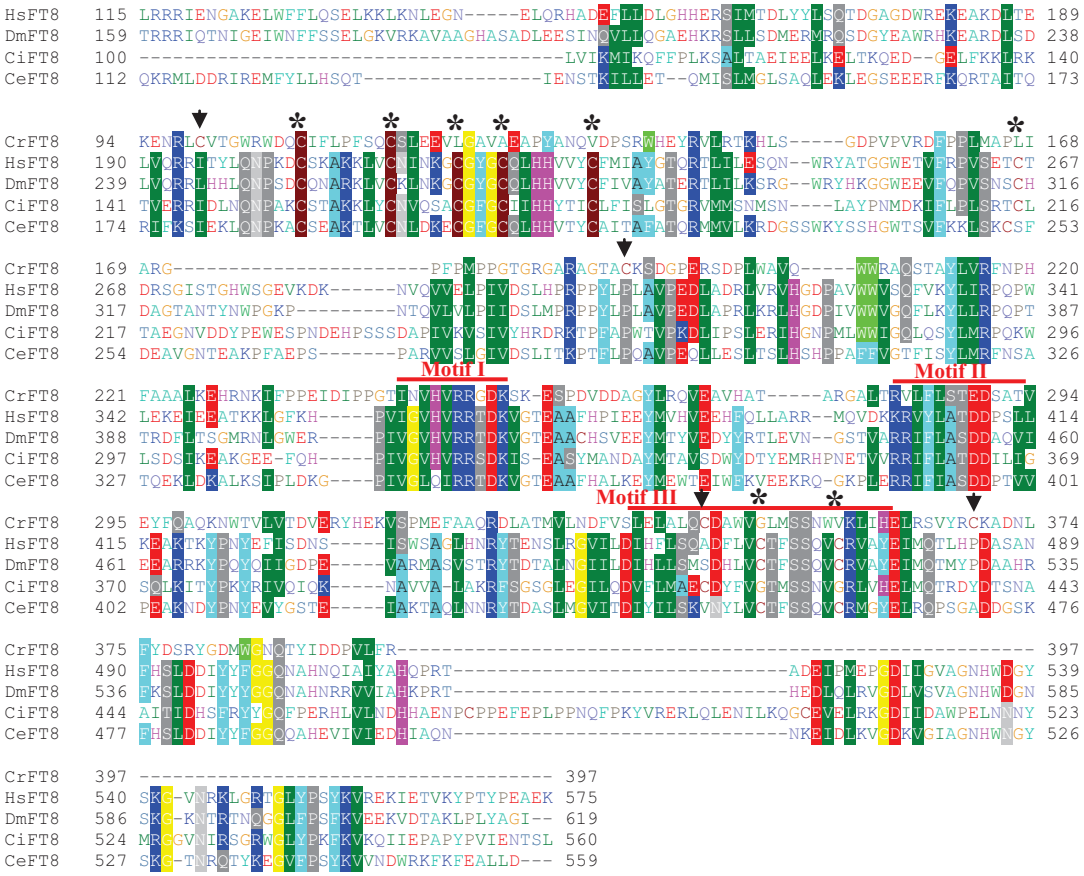


Figure 3.13: α(1,6)-fucosyltransferase (FT8) of Family GT23. A. Arrangement of the potential crft8 gene with 9 exons and 8 introns. Purple boxes are exons and arrows indicate the primers designed to PCR amplification. ***B. Alignment aminoacid sequence alignment of the Chlamydomonas CrFT8.*** Aminoacid sequence alignment of *C.reinhardtii* (CrFT8), *Homo sapiens* (HsFT8), *D.melanogaster* (DmFT8), *C.elegans* (CeFT8) *and C.intestinalis* (CiFT8). The motif I, motif II and motif III which are conserved in three fucosyltransferases α(1,2)-, α(1,6)- and protein O-fucosyltransferases, are indicated by red underlines. The asterisks indicate cysteine residues conserved in HsFT8, the arrows indicate the cysteines residues in CrFT8 in different position that HsFT8.

To check presence and expression of this putative gene, primers were designed to amplify an internal region of the putative gene *crft8* of *Chlamydomonas* (See Materials and Methods, section 2.4.5 and Figure 3.13A).

We isolated genomic DNA and total RNA from cells of *Chlamydomonas* wild strain cells growing in high CO_2 conditions and we have confirmed the presence of the sequence by PCR analysis (Figure 3.14A) as well as expression by semi-quantitative RT-PCR analysis (Figure 3.14B).

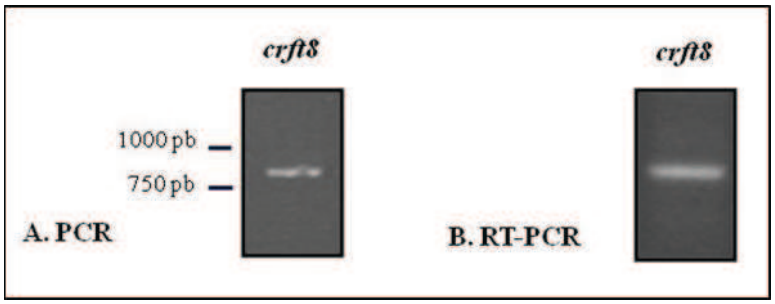


Figure 3.14: Presence and expression of the crft8 gen in C.reinhardtii of wild type cc 503 strain. **A:** PCR. cDNA was synthesized from total RNA isolated from *Chlamydomonas* wild type cells grown under high CO_2 conditions and a PCR amplification with specific primers FT8 R y FT8 F was performed. The PCR product was resolved on an agarose gel (1.5%). **B:** RT-PCR with specific primers FT8 R and FT8 F were performed. The PCR products were resolved on agarose gels (1.5%).

The next step was to identify the gene transcript, including the 5´-UTR and 3´-UTR regions as well as the initiation and termination codon. For this purpose we performed 5´ and 3´RACE techniques, as we explained previously in the Materials and Methods section (See section 2.4.7).

RACE products were cloned into pGEM T-Easy vector and we carried out the transformation in *E.coli* DH-5α, plasmid DNA isolation and digestion with restriction enzyme *EcoRI* was done to select those colonies that contained the vector with the appropriate cloned fragment.

Plasmidic DNA of colonies that contained the vector with an appropriate size fragment were sequenced. The results of sequencing allowed us to precise 5´and 3´ends of α(1,6)-fucosyltransferase gene of *Chlamydomonas*, as well as initiation and stop codons (Figure 3.15).

GCAGCTCCGGCCGCCATGGCGGCCGCGGGAATTCGATTAAGCAGTGGTATCAACGCAGAGTACGCGGGGTGTACCGAT
TGCATAGTTGTTGGATGTAACATTTGCTTGAAGCTGACCGCCTCGGTTATATGCAGCGGTCGCATGGATAATGCTGCT
AAAATTTAATTAGGAATTTGTTTCTGGCGCGGTGCAGGGTGTGTATTACGGCGCAGTCCAAAAAATGAGTCGGTGTTC
CTTCTTCTTCACCTTTGCCTTTTCCTTGCTCGCAATTAGCTCCGCCAAGTGGTACCGTAATTTCCTGTATCGTGGTGC
ATCCACCTGCTACAAGTTTAATACGGTTCATTTTAGGAACGCGACCGCGTGCTGGGACTCCGCCACGGTATGCTGATG
TGTGCAGTTCTGGATTAACTGGTTCAC**ATG**ATCGCACCCGGCGCGTATGTGGGCAACCGCGATGACCGGCCGGAGGAT
GGCAGGCAGCTCTACAACAGCGCGCTGGCGCAGCGCTTCATCTACCGCCACCAGAACCCGGCGGACTGCAGGTCCGCC
AAGTTCCTCGTCGCGGAGTTTGATGCCGAGGGTGCGTGCCCGTCCCGGGTGGGCATTGGCTCCACCATCCACATGATG
AGCTACGGCCTGACGCGGGCCATGATGATGGGGCGCGTGTACATCCACGAAGACGGCGGCCAGATATGGACGAAGGAA
AACCGGCTCTGCGTGACAGGCTGGCGCTGGGACCAGTGCATCTTCCTGCCCTTCTCGCAGTGCAGCCTGGAGGAGGTG
CTGGGCGCGGTGGCGGAGGCGCCCTACGCCAACCAGGTCGACCCCAGCCGCTGGCACGAGTACCGTGTCCTCCGGACC
AAGCACCTGTCCGGCGACCCAGTGCCTGTGCGCGACTTCCCGCCGCTCATGGCGCCACTCATCGCGCGCGGGCCCTTC
CCCATGCCGCCGGGCACGGGCCGCGGCGCGCGGGCCGGCACGGCGTGCAAGTCGGACGGCCCGGAGCGGTCCGACCCG
CTGTGGGCGGTGCAGTGGTGGCGGGCGCAGTCCACAGCCTACCTGGTGCGCTTCAACCCGCACTTCGCGGCCGCGTTG
AAGGAGCACCGGAACAAGATCTTCCCGCCCGAGATCGACATCCCGCCGGGCACCATCAACGTGCACGTACGGCGCGGC
GACAAATCTAAAG**AGTCCCCGGATGTGGACGACGCCGGCTACCTGCGGCAAGTGGAAGCCGTACACGCGACCGCCCGC**
GGCGCACTGACCCGCGTCCTGTTCCTGTCCACGGAGGACAGCGCCACGGTCGAGTACTTCCAGGCGCAGAAGAACTGG
ACGGTGCTGGTGACTGACGTGGAGCGCTACCACGAGAAGGTGTCGCCCATGGAGTTCGCGGCGCAGCGGGACCTGGCC
ACCATGGTGCTCAACGACTTTGTGAGCCTGGAGCTGGCGCTGCAGTGCGACGCCTGGGTGGGCCTGATGTCCTCCAAC
TGGGTGAAGCTCATCCACGAGCTGCGGTCCGTGTACCGCTGCAAGGCCGACAACCTGTTCTACGACTCACG**GTACGGT**
GACATGTGGGGCAACCAGACATACATCGACGACCCCGTTCTATTCCGC**TAG**CTTGCATTTCCATGCGCTAGCTGGTGG
CTTCTCCTGCTGGCTAGTGGCTCCACCTGCTGCCTCCACGTGCTGCATGCACGTGCTGGCTCCACTGGCCGAAGGCAC
ACCGGCATCACGCGCACCGGGTGCACCCGCATGGGGTGAGGCCGATGAGCAGCGGAAGCAGGGCGGCGCGCCGTGTCC
GGATCACAGCCAACGGGCTATGGATGGAGGCCGCACTGAGCGGGCCAGTCCGCCCGTTGTGTACGTGATGCTGTGCCG
CATACACAGGGCTGGGGGCTTGCAAAGTAACGCATGCCCCCAGTCAAAGCATGTTCCCCCGGCACCGTCCAACGCACG
AGCTAGGAGACGTGGGAGCGCACATTCAAGGGATTGACGATTTGACATGCTGAAGGAGACTTGTATGCGGTATTGGCA

TGCTGGAGGAAACCTGGAGTAGGGGTGTGCAGTGTGTTTTTGTATGTGAGGTGCTGGTTACCCAAGTGGTGCGCACTC
TATTGCCCTGTTGCGTGCCTAACTGCCACATGATAAGCGGACGCCCGCCAAGTGTCACTGGCGGGCGGTGCCCTGTAC
AAACCGACCTCGG

Figure 3.15: cDNA sequence of Chlamydomonas α(1,6)-fucosytransferase gene with the 5´-UTR and 3´-UTR. *Exons are marked in different colors and the ends 5´-UTR and 3´-UTR are colored grey. The initiation and stop codons are highlighted.*

If we found that *crft8* gene is expressed, we should be able to detect α(1,6)-fucose residues in *Chlamydomonas* glycoproteins. For that, we used the lectin AAL (biotinylated *Aleuria aurantia* Lectin) that recognizes α(1,6)-fucose residues. To test this, we did affinoblot analysis using AAL lectin (See Materials and Methods, section 2.5.4) with 10 µg protein of total cells extracts (TE) isolated from cells of *Chlamydomonas* cc503 wild type strain growing under high CO_2 .We used as a positive control, 10 µg protein of total cells extracts from *D.melanogaster* and as negative controls 10 µg protein of total cells extracts from *A.thaliana* wild type strain, *cgl1* mutant of *Arabidopsis thaliana* and ft11/ft12 mutant of *A.thaliana*.

Figure 3.16 shows the presence of α(1,6)-fucose residues in the endogenous algal N-glycoproteins so *crft8* gene of *Chlamydomonas* seems to be a functional gene.

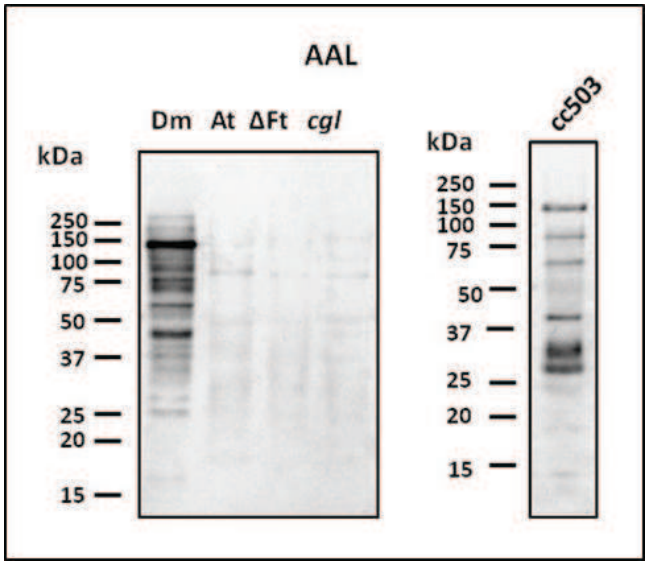


Figure 3.16: Presence of α(1,6)-fucose residues in the endogenous alga N-glycoproteins. Affinoblot using AAL lectin (biotinylated Aleuria aurantia Lectin) Dm: *D.melanogaster;* At: *A.thaliana*; ΔFT: *ft*11/*ft*12 mutant of *A.thaliana; cgl*1: *GnTI* mutant of *A.thaliana;* cc503: wild type strain of *C.reinhardtii.*

These results could be an evidence to confirm that α(1,6)-fucose residues might be blocking Endo H action on the N-glycans linked to endogenous N-glycoproteins in *Chlamydomonas*. However, the latter has to be further confirmed by different approaches, as N-glycan analysis or expression of *crft8* gene in a heterologous system. Other possibility will be to do silencing of *crft8* gene by artificial microRNA and to analyze the sensitivity to Endo H of N-glycans in the transgenic lines.

CHAPTER 4:

Analyses of L23 mutant of *Chlamydomonas reinhardtii* defective in N-glycosylation of glycoproteins.

Professor Robert A. Bloodgood and coworkers, in 1987, as part of their work focused on the study of the structure and function of flagella in *Chlamydomonas*, generated and characterized a mutant named L23.

The mutant strain L23 was selected after chemical mutagenesis of cc1036 PF-18 cells by using fluorescence-activated cell sorting (Bloodgood *et al.,* 1987). The parental strain cc1036 PF-18 is defective in the central pair microtubules of flagella and therefore its motility is completely impaired (Adam *et al*., 1981). L23 was selected because it did not bind to a monoclonal antibody, designated FMG-1. The FMG-1 monoclonal antibody was raised against a carbohydrate epitope associated with the exposed surface of the flagellar membrane and the cell wall; in each case, this epitope was associated with unique proteins**.**

Bloodgood *et al*. (1987) concluded that FMG-1 monoclonal antibody should recognize a terminal sugar on an N-linked complex carbohydrate side chain. Baenziger and Fiete (1979) had shown that the absence of some or all terminal sugars on a mammalian N-linked complex carbohydrate-type glycoprotein results in a dramatic increase in the association to Concanavalin A. Bloodgood *et al.,* (1987) showed that L23 exhibited a significant increase in concanavalin A binding as well as a dramatic loss in binding to the anti-carbohydrate monoclonal antibody FMG-1. The latter were compatible with a defect in the glycosyltransferase necessary for the addition of the terminal sugar necessary for the binding of FMG-1 monoclonal antibody or a defect in one of the processing enzymes (such as a glucosidase or mannosidase) necessary for trimming the high mannose configuration before terminal glycosylation can occur. It seemed therefore, that this mutant was affected in the N-glycan biosynthesis of endogenous proteins and it would be useful in the study of this process in *Chlamydomonas.*

4.1 Analysis of N-glycosylation pathway in L23 mutant of *C.reinhardtii*.

As far as we know, L23 is the only mutant affected in the N-glycosilation pathway in *Chlamydomonas*, so we decided to analyze it in order to shed light on N-glycosilation pathway and on the structure and composition of N-glycans in *Chlamydomonas reinhardtii*.

In order to confirm the information that we already had about L23 mutant, we began performing immunoblot and affinoblot analyses comparing total protein extracts from L23 mutant with total protein extracts from cc503 (wild type) and cc1036 strains (parental strain) of *Chlamydomonas*. To test specificity of the used antibodies and lectins we used as control total protein extracts from *D.melanogaster*, *A.thaliana* wild type as well, as the "complex glycan less" *cgl1* mutant and the "α(1,3)fucose less" *ft11/ft12* mutant already mentioned above.

Figure 4.1 shows the Coomassie blue staining geles we performed as loading control.

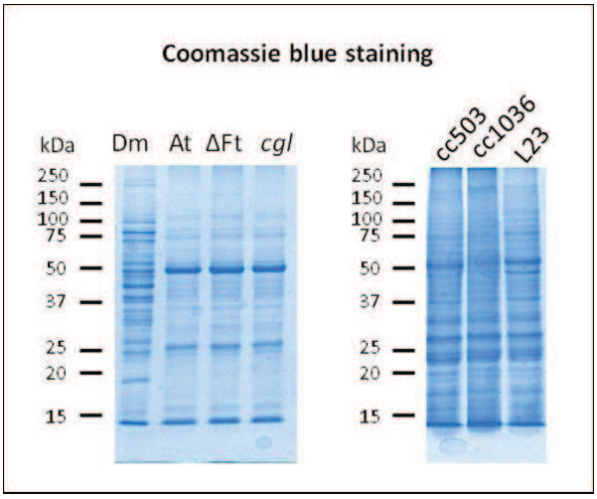


Figure 4.1: Coomassie blue staining. 10 µg of protein of total cell extracts were separated by electrophoresis on 12% acrylamide gels and stained with Comassie blue. Dm: *D.melanogaster;* At: *A.thaliana*; ΔFT: ft11/ft12 mutant of *A.thaliana; cgl*1: *GnTI* mutant of *A.thaliana;* cc503: wild type strain of *C.reinhardtii;* cc1036: parental strain of L23 mutant; L23: mutant of *C.reinhardtii.*

Then, we performed inmunobloting analysis with the antibody raised against FMG-1, kindly donated by Prof. Bloodgood, which recognizes a carbohydrate epitope on the major flagellar membrane glycoprotein. Figure 4.2 shows that this epitope is absent in the L23 mutant strain, but it is detected in endogenous glycoproteins of the wild strain cc503 and the parental strain cc1036 of the L23 mutant. It should be noted that FMG-1 antibody did not recognize proteins in any of the organisms used as control. Therefore the carbohydrate epitope recognized by FMG1 antibody is specific of *Chlamydomonas*.

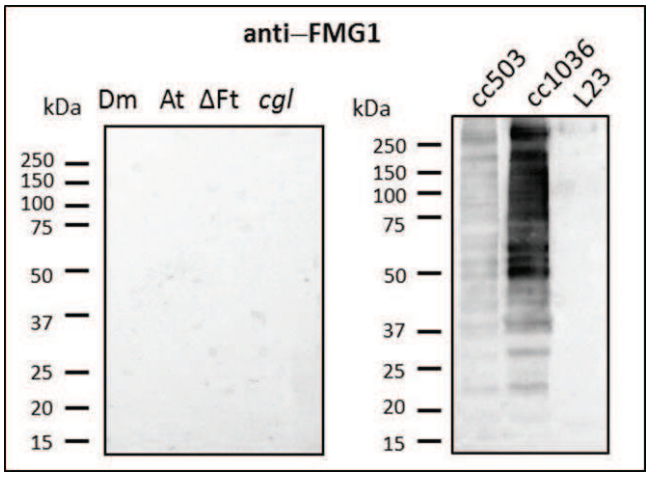


Figure 4.2: Analysis of N-glycosylation in the mutant L23. 10 µg of protein of total cell extracts were separated by electrophoresis on 12% acrylamide gels and transference onto nitrocellulose membranes was made. Immunodetection was performed with a primary antibody raised against carbohydrate epitope on the major flagellar membrane glycoprotein (FMG-1) and a secondary antibody coupled to horseradish peroxidase. Dm: *D.melanogaster;* At: *A.thaliana*; ΔFT: ft11/ft12 mutant of *A.thaliana; cgl*1: *GnTI* mutant of *A.thaliana;* cc503: wild type strain of *C.reinhardtii;* cc1036: parental of L23 mutant; L23: mutant of *C.reinhardtii.*

High-mannose type N-glycans were detected by affinoblot analysis using Con A lectin. We observed in Figure 4.3 that L23 endogenous proteins present a higher amount of high-mannose type N-glycans than the wild type strain, but similar levels of its parental strain. These data corroborates those obtained by Dr. R. Bloodgood, showing that the N-glycans associated to the glycoproteins of L23 mutant has higher content of mannose and therefore a higher affinity to Con A. On the other hand, Figure 4.3 again confirm that *cgl1* mutant of *Arabidopsis* has a higher amount of high-mannose type N-glycans than both wild type and *ft11/ft12* double mutant.

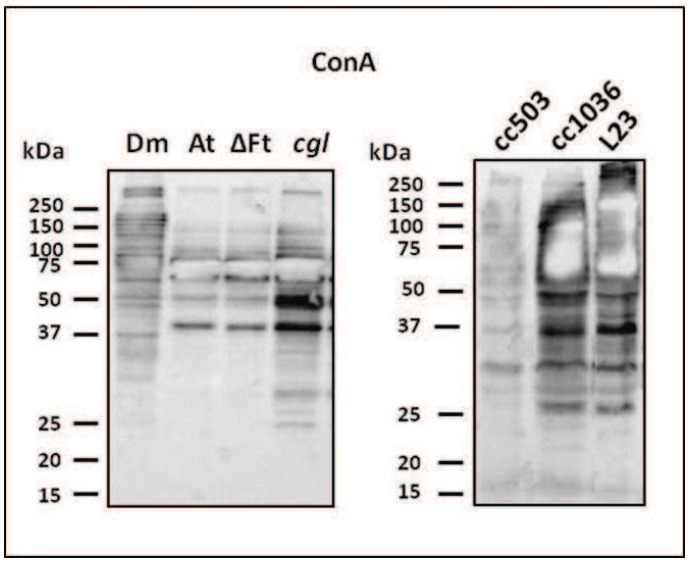


Figure 4.3: ***Analysis of N-glycosylation in the mutant L23.*** Affinoblot based on the affinity of the N-glycans to Con A lectine was made according to conditions already explained in Figure 4.2. Dm: *D.melanogaster;* At: *A.thaliana*; ΔFT: *ft11/ft12* mutant of *A.thaliana; cgl1*: GnTI mutant of *A.thaliana;* cc503: wild type strain of *C.reinhardtii.*

To know if N-glycan of L23 mutant present α(1,3)-fucose and β(1,2)-xylose residues typical of complex type N-glycans, as *Chlamydomonas* wild type strain, we performed immunoblot analyses with antibodies raised against α(1,3)-fucose and β (1,2)-xylose residues (Figure 4.4). Figure 4.4A shows that the levels of fucosylation in the L23 mutant and cc1036 strain of *Chlamydomonas* are the same or slightly higher than in the wild type strain cc503. As we expected, α(1,3)-fucose antibody recognized fucosylated proteins of *D. melanogaster*, and *A. thaliana* wild type strain, but not in A. *thaliana* mutants (Figure 4.4A). On the other hand, Figure 4.4B shows that the parental strain cc1036 and L23 glycoproteins present more β(1,2)-xilose residues when compared with *C.reinhardtii* wild type strain cc503 (Figure 4.4B). As expected, β(1,2)-xilose antibody recognized *A. thaliana* wild type and *ft11/ft12* mutant glicoproteins, but not those of *D. melanogaster* and *cgl1* mutant of *A. thaliana.*

All these data could indicate that L23 mutant is not defective either in CrFT1 *or* CrXylT.

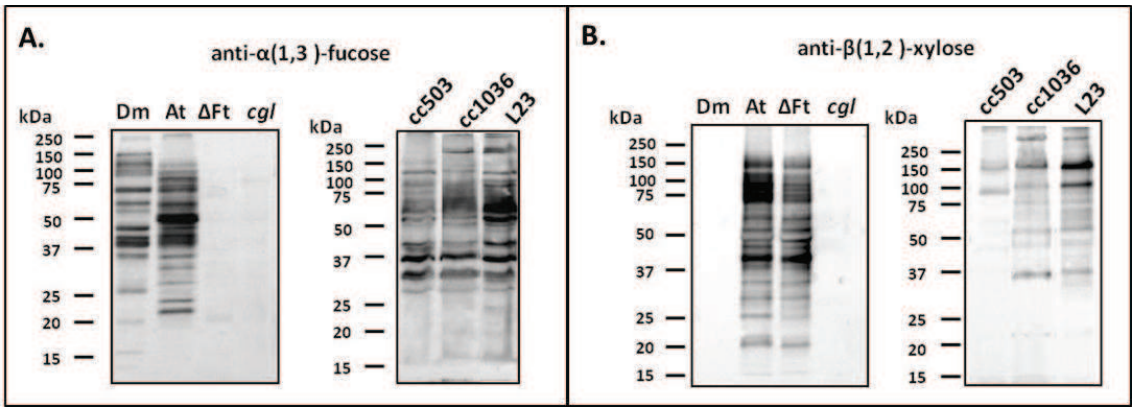


Figure 4.4: Glycosylation pattern of proteins in L23 mutant. 10 µg of protein of total cell extracts were separated by electrophoresis on 12% acrylamide gels and transference onto nitrocellulose membranes was made**. A**. Immunodetection was performed with a primary antibody raised against α(1,3)-fucose residues β(1,2)-xylose residues and a secondary antibody coupled to horseradish peroxidase. **B.** Immunodetection was performed with a primary antibody raised against β(1,2)-xylose residues and a secondary antibody coupled to horseradish peroxidase. Dm: *D.melanogaster;* At: *A.thaliana*; ΔFT: ft11/ft12 mutant of *A.thaliana; cgl1*: *GnTI* mutant of *A.thaliana;* cc503: wild type strain of *C.reinhardtii;* cc1036: parental of L23 mutant; L23: mutant of *C.reinhardtii.*

Finally, we performed affinoblot analysis using AAL lectin (biotinylated *Aleuria aurantia* Lectin) to check for the presence of α(1,6)-fucose residues in the glycoproteins of the three strains under analysis (Figure 4.5). First of all, it has to be noted that α(1,6)-fucose detection by AAL lectin is specific for this residue and linkage since, as it was expected, only recognized glycoproteins from *D.melanogaster* and not from *A.thaliana* samples which lack α(1,6)-fucose residues, as all plant N-glycoproteins. In addition, Figure 4.5 shows that L23 mutant and cc1036 parental strain present glycoproteins with α(1,6)-fucose residues as the wild type strain of *C.reinhardtii*.

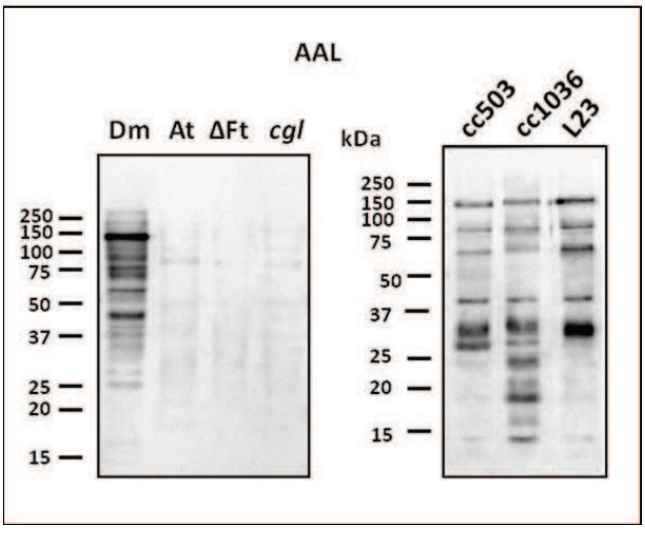


Figure 4.5: Glycosylation* of *the endogenous* proteins *in the L23 mutant. **A.** Affinoblot using AAL lectin (biotinylated *Aleuria aurantia* Lectin) was made according to conditions already explained in Figure 4.2. Dm: *D.melanogaster;* At: *A.thaliana*; ΔFT: ft11/ft12 mutant of *A.thaliana; cgl1*: *GnTI* mutant of *A.thaliana;* cc503: wild type strain of *C.reinhardtii.*

The higher content of mannose in L23 glycoproteins, led us to consider the possibility that the mutant L23 is somehow impaired in α-Mannosidase I. Therefore, we decided to analyze the expression of *crmanI* gene in the L23 mutant. At the same time, we studied the expression of the other genes involved in N-glycosylation pathway that we already found to be expressed in *Chlamydomonas* wild type strain.

A complete semi-quantitative RT-PCR analysis was performed to check the expression of the genes that are implicated in *Chlamydomonas* N-glycosilation pathway. *crmanI* expression was checked using specific primers ManIF and ManIR (see Materials and Methods, section 2.4.5). We found that whereas in wild type strain *crmanI* is expressed (we amplified a fragment of the expected size), the gene is not expressed in L23 mutant strain (Figure 4.6). However, the expression of Mannosidase II (c*rmanII*), β(1,2)-xylosyltransferase (c*rxylt*), α1,3-fucosyltransferase (c*rft1*) and α(1,6)-fucosyltransferase *(crft8)* were not altered in L23 mutant (Figure 4.6). These results confirmed the data obtained with the immunoblots and affinoblots analyses (Figures 4.3, 4.4 and 4.5) and indicate that L23 mutant is defective in *crmanI* expression as well as in α-Mannosidase I activity.

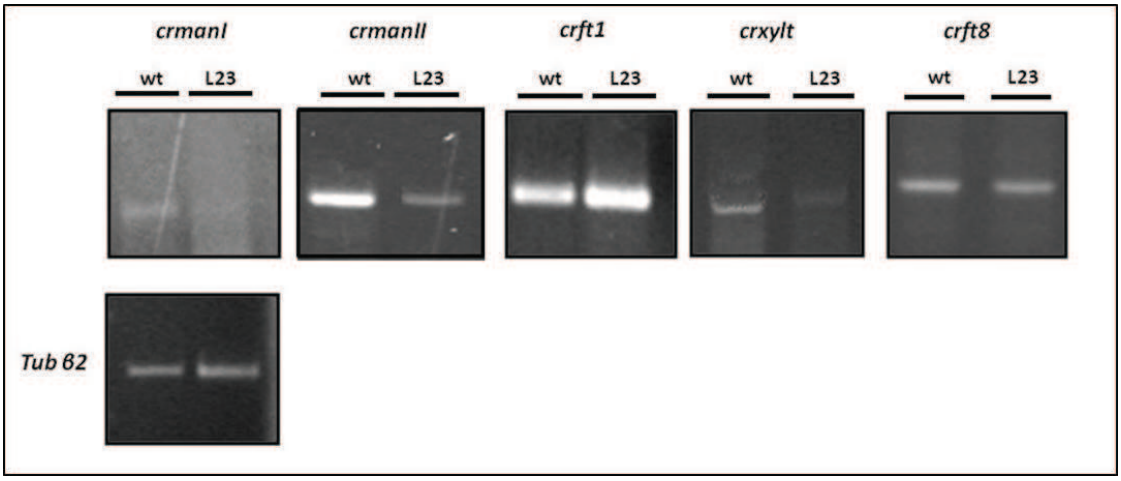


Figure 4.6: Expression of crmanI, crmanII, crft1 and crxylt genes in C.reinhardtii wild type cc 503 strain and L23 mutant. Semi-quantitative RT-PCR analyses with specific primers (See Materials and Methods, section 2.4.5) were performed. The PCR products were resolved on agarose gels (1.5%).

In addition, these data also indicate that CrFT1 activity is not only independent of GnT I as we previously demonstrated (see Results Chapter 3), but does seem to require CrManI action either.

In order to determine whether the lack of expression of *crmanI* observed in L23 was due to a failure in expression or in the genome, we decided to analyze the genomic sequence and look for a possible mutation.

Genomic DNA from *Chlamydomonas* wild type strain and L23 mutant cells growing under high CO_2 conditions was isolated. We performed PCR using the specific primers α-ManIR and α-ManIF which amplify an internal region of *crmanI* (see figure

2.3 Chapter 1 of the Results section) under a gradient of annealing temperatures (T: 58, 56.4, 53.2 and 50 ° C) (Figure 4.7).

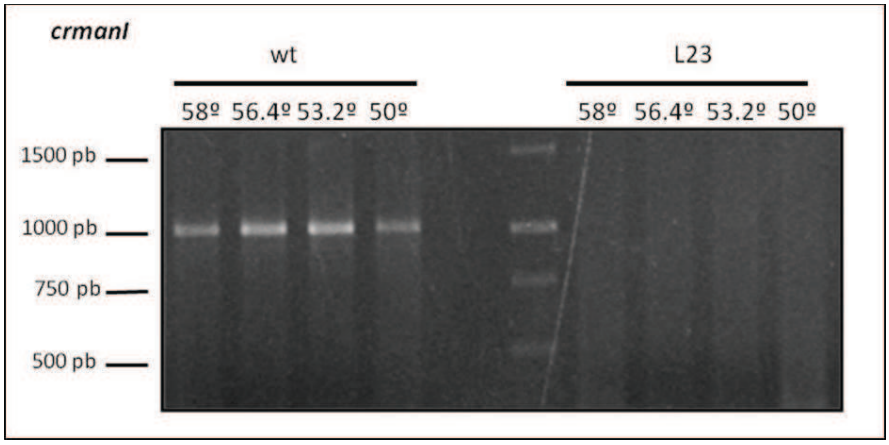


Figure 4.7: PCR analysis of gene coding for α(1,2)-Mannosidase (crmanI) of L23 mutant of Chlamydomonas reinhardtii. DNAg was synthesized from total RNA isolated from *Chlamydomonas* wild type cells grown under high CO_2 conditions and a PCR amplification with specific primers α-ManIR and α-ManIF was performed. The PCR product was resolved on an agarose gel (1.5%).

As shown in Figure 4.7, L23 mutant presents a deletion in at least part of the coding sequence of the gene coding for α-Mannosidase I.

A genomic PCR analysis of the other four genes found to be involved in Golgi *N*-glycan maturation in *Chlamydomonas* was done. Figure 4.8 shows that *C.reinhardii* genes for Mannosidase II (c*rmanII*), β(1,2)-xylosyltransferase (c*rxylt*), α1,3-fucosyltransferase (c*rft1*) and α1,6-fucosyltransferase (c*rft8*) are present in L23 mutant genome.

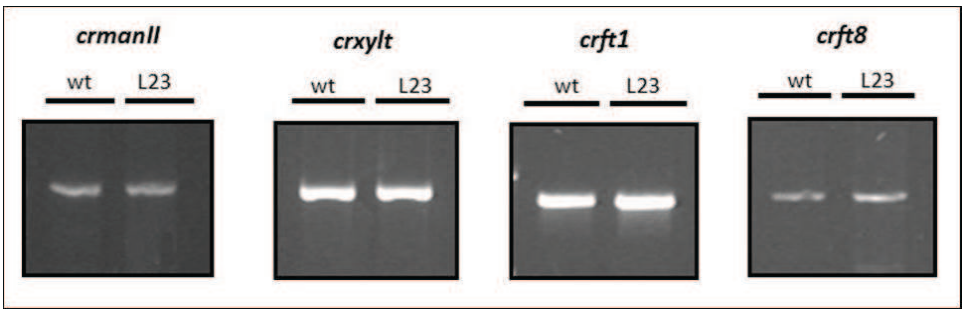


***Figure 4.8: Presence of crmanII, crxylt, crft1 and crft8 genes in L23 mutant* PCR analyses.** DNAg was synthesized from total RNA isolated from *Chlamydomonas* wild type cells grown under high CO_2 conditions and a PCR amplification with specific primers (See Materials and Methods, section 2.4.5) were performed. The PCR products were resolved on agarose gels (1.5%).

The phenotypic defect observed in L23 mutant, higher content in high mannose type N-glycoproteins is due to the total or partial absence of the coding sequence of the *crmanI*.

Based in all the results obtained with L23 mutant we can assume that the addition of α(1,3)-fucose, is not only is independent of GnT I, but also is independent

the elimination of terminal mannoses. By contrast, addition of the epitope recognized by antibody FMG-1 needs the prior action of α-Man I.

4.2 Analyses of L23 N-glycans of *Chlamydomonas* with commercial glycosidases.

As we did for wild type strain in Chapter 3, we analyzed the sensitivity of L23 mutant N-glycans to two commercial endoglycosidases, Endo H and PNGase F. For this case we analyzed separately the periplasmic fraction in order to discriminate any possible difference between samples as being caused by N-glycan composition and not by flagellar protein deficiency intrinsic to this mutant.

Total cell extracts (TE) and periplasmic fraction (FP) were isolated from *Chlamydomonas* L23 mutant cells grown under high CO_2 conditions, were treated for 24h with Endo H and PNGase F enzymes. After digestion, the treated samples were separated by acrylamide gel electrophoresis followed by a transference to nitrocellulose membranes to perform an Affinoblot assay with Con A lectine (Figure 4.9A) and immunodetection with antibody against α(1,3)-fucose residues (Figure 4.9B).

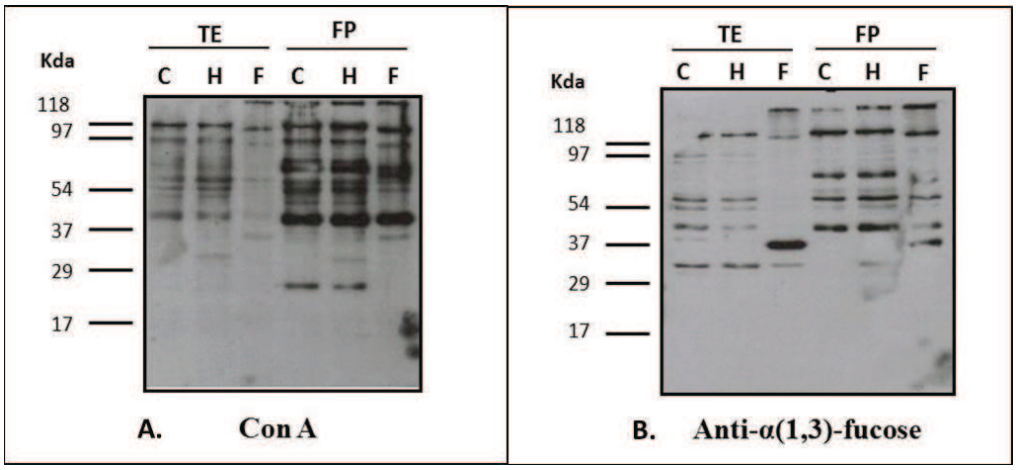


Figure 4.9: Sensitivity of L23 N-glycoproteins to endoglycosidases Endo H and PNGase F. Total cell extracts (TE) and periplasmic fractions (FP) from *Chlamydomonas* L23 mutant cells grown in high CO_2 conditions. Samples were digested with Endo-H and PNGase F for 24h and 2 µg of chlorophyll were separated by electrophoresis on 10% acrylamide gels and transference onto nitrocellulose membranes was made**. A.** Affinoblot based on the affinity of the N-glycans to Con A**. B.** Immunodetection was performed with a primary antibody raised against α(1,3)-fucose residues and a secondary antibody coupled to horseradish peroxidase. TE: Total Extract; FP: Periplasmic fraction; C: Control; H: Endo-H; F: PNGase F.

Figure 4.9 shows, similar to what we found for cc503 wild type train, that the N-glycans anchored to the algal N-glycoproteins in L23 mutant keep resistant to Endo H digestion (H) which means that, despite of the no trimming of terminal mannoses in this mutant, the N-glycan still contain some type of residue blocking the action of this glycosidase. On the other hand, L23 N-glycoproteins are sensitive to the action of PNGase F (F), further confirming that *Chlamydomonas* CrFT1 does not add α(1,3)-fucose residues to the proximal GlcNAc of the quitobiose core, unlike plant α(1,3)-fucosyltransferases do. In addition, these data indicate that CrFT1 is not only

independent of GnT I but does not require α(1,2)-mannosidase I activity for its action either.

4.3 Future work with L23 mutant.

In order to confirm that phenotype of L23 mutant is only due to *crmanI* deletion, we want to do a classic complementation study in L23 mutant. Briefly, we need to amplify and clone the complete coding region of wild type *crmanI* gene and introduce the gene in L23 mutant using biolistic techniques. As we "give" a wild type copy of the gene to L23 mutant, we should be able to detect FMG1 epitope and a decrease in high mannose type N-glycans. In addition we should be able to amplify *crmanI* gene by RT-PCR analysis.

On the other hand, silencing of *crmanI* gene based on amiRNA in *Chlamydomonas* wild type strain cc425 agr2 cw15 is under progress. The silenced lines should present, in addition to a lower expression of *crmanI*, the same phenotype than L23 mutant with higher amounts of high mannose type N-glycans and positive detection of N-glycoproteins with FMG-1 monoclonal antibody.

CHAPTER 5:

Analysis of the N-glycan structure linked to endogenous algal glycoproteins in *Chlamydomonas reinhardtii.*

Finally, we focused our efforts on the analysis of the N-glycan structure linked to endogenous algal glycoproteins, our last objective in this work. The objective was to define the detailed structure of the N-glycans linked to the *Chlamydomonas* endogenous glycoproteins. This work was mainly made by the French group headed by Dr. Muriel Bardor and Prof. Patrice Lerouge at the University of Rouen, which is a pioneer group in the analysis of N-glycoproteins of plants and algae using the Mass Spectrometryc techniques. To accomplish this part of the work I spent three months (October to December 2010) in their laboratory at the University of Rouen to learn and participate in the N-glycan characterization of the different strains of *Chlamydomonas* used in this study.

As we explained in Materials and Methods (Section 2.1.3) we started by working with the soluble endogenous proteins from the different *Chlamydomonas* strains (cc503 wild type stain, cc1036 and L23 mutant) and their N-glycosylation profiles were compared. We used a novel approach of N-glycan release and we had a robust method for preparing N-glycans from *Chlamydomonas*. We deglycosylated the protein extract either using peptide N-glycosidase F (PNGase F) or separated 5 mg of proteins on a SDS-PAGE prior to a trypsin and PNGase A digestion. Then, the following steps are quite similar and required purification of the free N-glycans and their analysis by MALDI-TOF mass spectrometry (MS) or tandem mass spectrometry (MS-MS) after labeling with the 2-AB fluorescent tag (2 amino-benzamide).

The experiments performed after PNGase F digestion demonstrated that *Chlamydomonas* N-glycans from cc503 wild type strain and cc1036 (the parental strain of L23 mutant) present oligomannosides from Man-5 to Man-2 (Figure 5.1). The structure of the Man-5 oligomannoside has been confirmed by MS-MS and by chromatography in comparison with a Man-5 standard. We also found another population of N-glycans substituted by one or two pentoses and bearing either a fucosyl residue or a methylated mannose (Figure 5.1). These results have been confirmed by alpha mannosidase treatment (Figure 5.2) which is able to shift the masses of all the oligomannoside N-glycans from Man-5 to Man-2 to a single Man-1 N-glycan. The second population bearing one or two xylosyl residues was not completely sensitive to the alpha-mannosidase treatment confirming the presence of substitution on these oligomannosides.

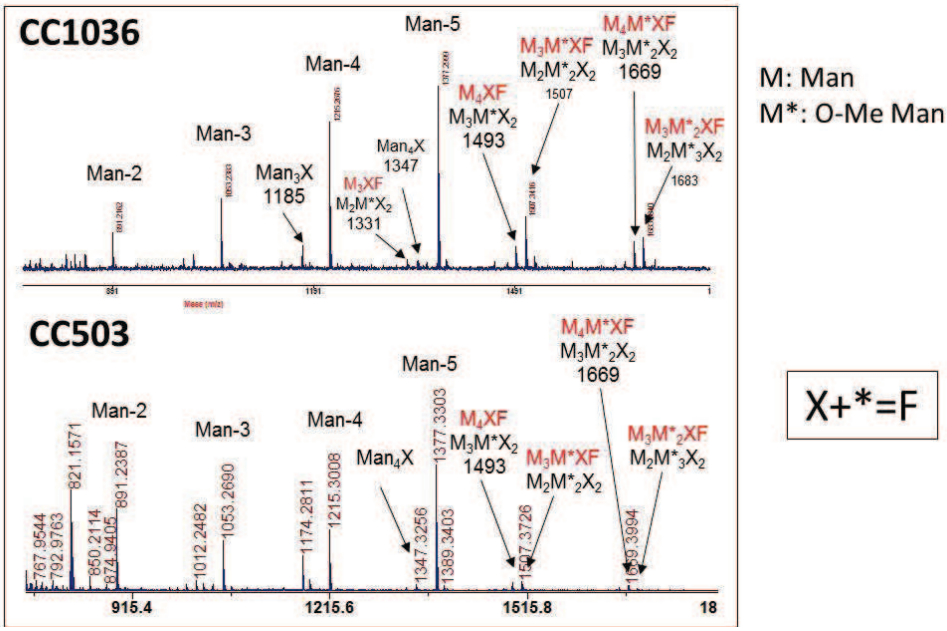


Figure 5.1: MALDI-TOF mass spectra of N-linked glycans from Chlamydomonas cc1036 and cc503 wild type strain proteins released by PNGase F and labeled with 2-aminobenzamide. Man or M: Mannose; X: xylose; F: fucose; asterisk represent the methyl group substitution.

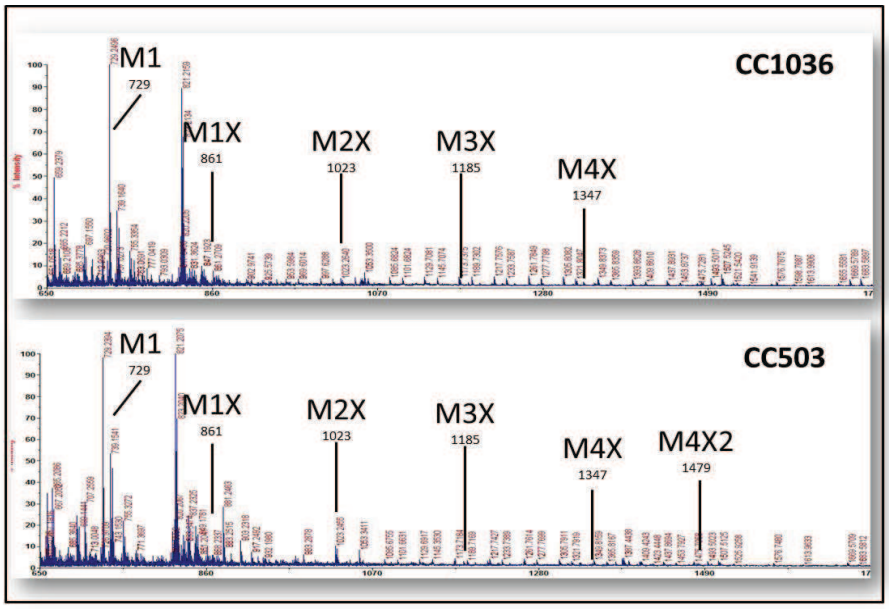


Figure 5.2: MALDI-TOF mass spectrum of N-linked glycans released from Chlamydomonas reinhardtii CC1036 and cc503 proteins by PNGase F and labeled with 2-aminobenzamine (2-AB) after 72h alpha-mannosidase treatment.

The PNGase A experiments gave similar N-glycan profiles for both strains to those obtained after PNGase F digestion (*Data not shown*).

In order to distinguish between the methylation and the fucose substitutions, we did a permethylation treatment on the above mentioned samples. The permethylation treatment, which exchanges any free OH groups from the N-glycans by methyl groups, lead us to a mass shift of 14 Da, thus confirming the hypothesis of the presence of a methyl-mannose (Figure 5.3). Only the N-glycans from cc1036 were found to be

substituted by both a xylosyl and a fucosyl residues (Man3XF, Figure 5.3). However, N-glycans from cc503 apparently harbored only methylmannoses and 1 or 2 xylosyl residues (Figure 5.4). Furthermore, preliminary gas chromatography analysis had shown the presence of 6-O-methylmannose and xylose in the monosaccharide composition obtained from *Chlamydomonas* N-glycans. We are currently repeating this gas chromatography analysis and will extend it to confirm the presence of xylose, methylmannose as well as fucose in the monosaccharide composition of the N-glycans from *Chlamydomonas* cc1036 and cc503.

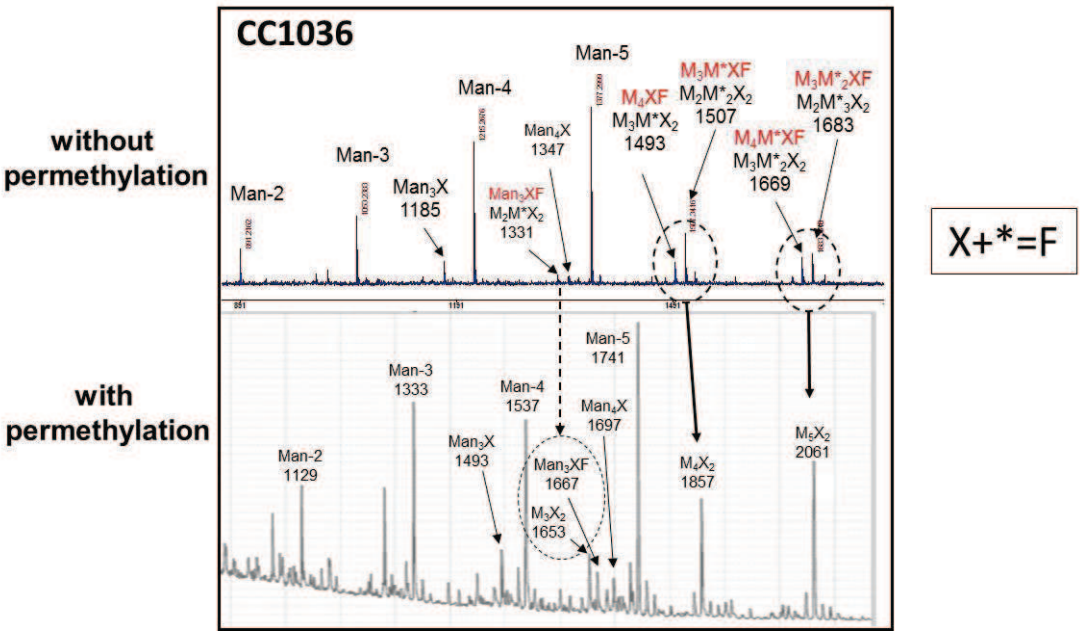


Figure 5.3: Comparative analyses of cc1036 N-glycans MS profiles obtained before and after permethylation. Man or M: mannose; X: xylose; F: Fucose; asterisk represent the methylation.

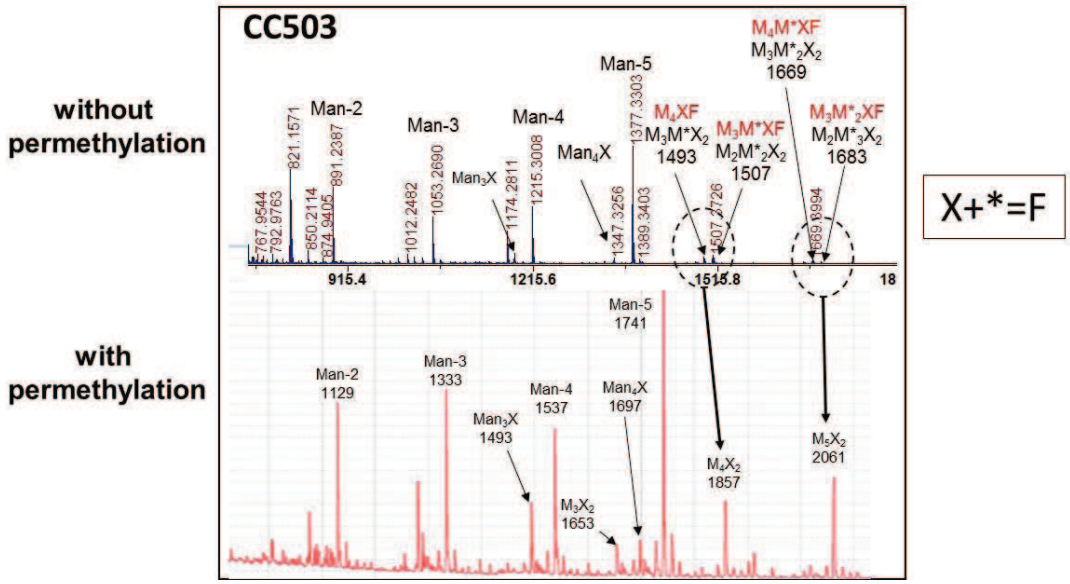


Figure 5.4: Comparative analyses of cc503 N-glycans MS profiles obtained before and after permethylation. Man or M: mannose; X: xylose; F: Fucose; asterisk represent the methylation.

Complementary experiments of MS-MS performed after PNGase A digestion, 2-AB labeling and permethlylation of N-glycans allowed us to position one xylosyl residue on the first mannose from the core in the N-glycans and the second one in terminal position of the oligomannosides (Figure 5.5). Now, we need to determine the linkage of those xylosyl residues. Based on our western blot analysis using specific core β(1,2)-xylose antibodies, it is likely that the xylose from the core is linked in β(1,2)-. Moreover, MS-MS experiment performed on the remaining Man1XylGlcNac2 2AB-labeled N-glycans after the mannosidase treatment confirm the presence of the xylose in β(1,2)- on the core mannose.

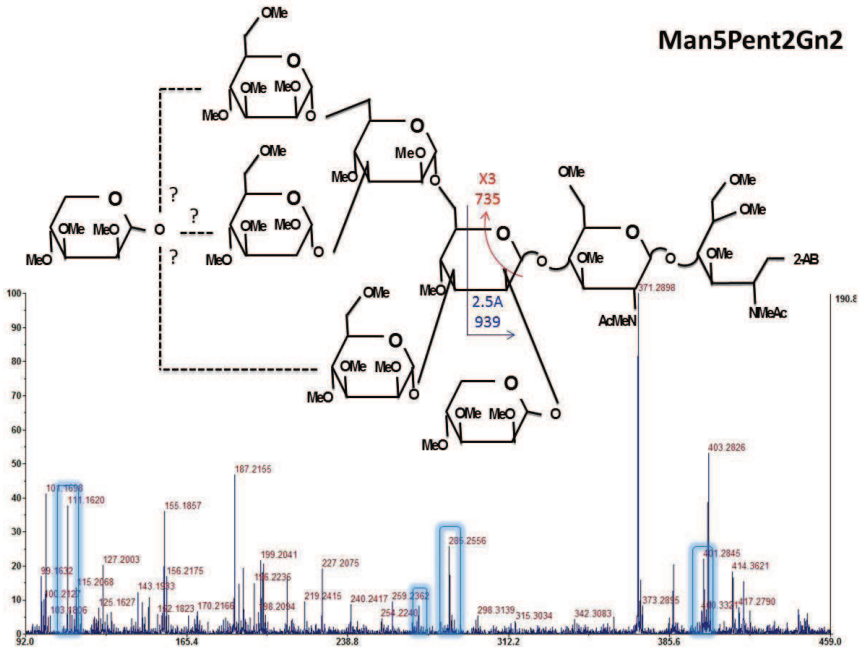


Figure 5.5: MALDI-TOF MS-MS analyses of N-linked glycans from Chlamydomonas cc503 wild type strains proteins released by PNGase A, labeled with 2-aminobenzamide and permethylated.

Despite the presence of two putative fucosyltransferases in the genome of *Chlamydomonas reinhardtii*, a low abundance of N-glycans bearing fucosyl residues had been found in the structural analyses. We reasoned that the latter could be due to the fact that we only analyzed soluble endogenous proteins and therefore we decided to extend this study and compare the results obtained for N-glycan analysis from soluble proteins to those we will obtain from internal membranes and cell wall proteins. This is currently under progress and should be completed in the next months.

The N-glycan structural analysis of *Chlamydomonas* plastid glycoproteins still need to be performed. This analysis will be quite challenging because of the small quantity of such glycoproteins in the chloroplast.

In parallel to the N-glycan analyses of glycoproteins from cc1036 and cc503 *Chlamydomonas* strains, we have also characterized the N-glycans from L23 mutant originated from cc1036 strain.

Using the same methodologies, we were able to demonstrate that L23 is bearing a higher number of mannose residues in its oligomannosides as compared to those from cc1036 (Figure 5.6) or cc503 wild type strain. Indeed, the N-glycan structures observed for the L23 mutant are going from Man-5 to Man-9 which is in agreement with the mutation in *crmanI* gene we have previously demonstrated. These oligomannosides are also bearing one or two xylosyl residues as observed for cc1036 strain, but are not methylated (Figure 5.6). These results have been confirmed by alpha mannosidase treatment (Figure 5.7) which shifted the masses of all the oligomannoside N-glycans from Man-6 and Man-5 to a single Man-1 N-glycan, rending a second population bearing one or two xylosyl residues which was not completely sensitive to the alpha-mannosidase treatment as was also found for cc503 and cc1036 N-glycans after alpha-mannosidase treatment (Figure 5.2).

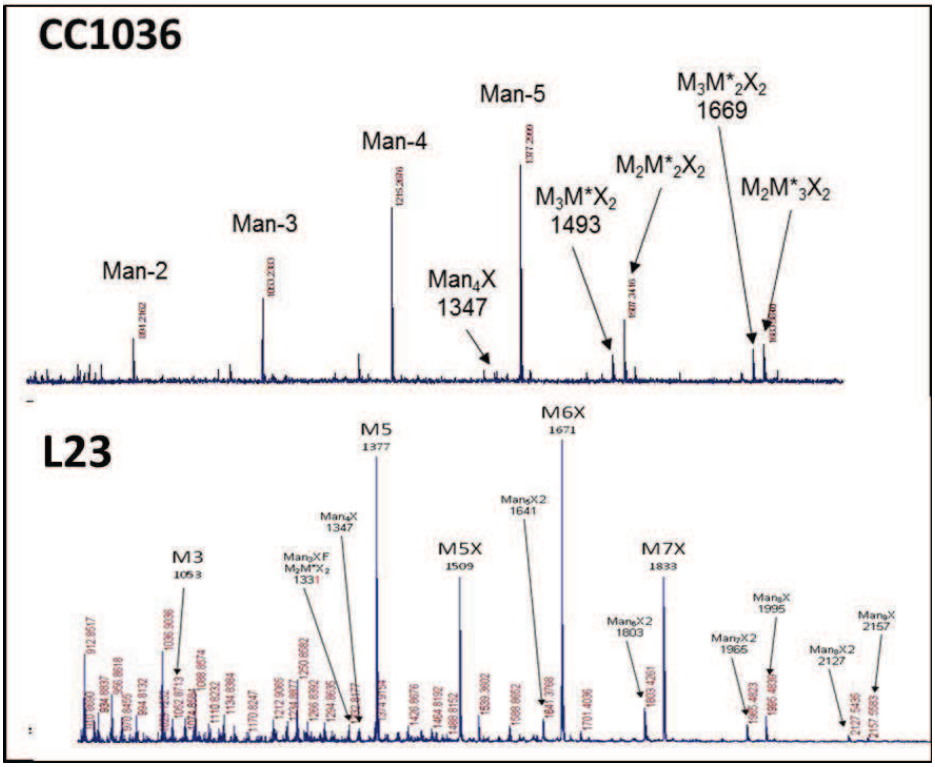


Figure 5.6: MALDI-TOF MS comparative analysis of cc1036 and L23 N-glycans profiles obtained after PNGAse F or A digestion and labeling with 2-AB. Man or M: mannose; X: xylose. Asterisk represent the methylation.

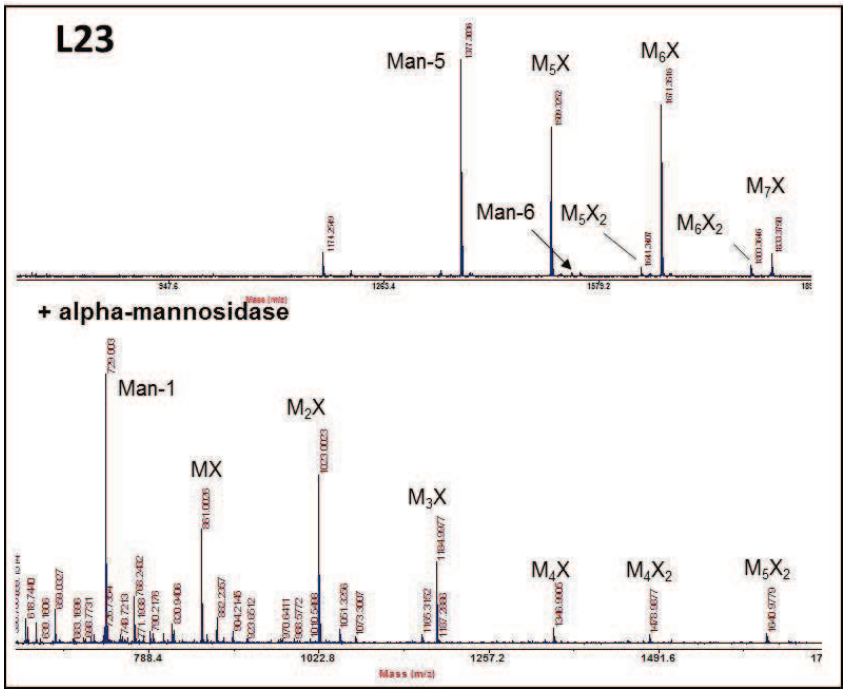


Figure 5.7: MALDI-TOF mass spectrum of N-linked glycans released from Chlamydomonas reinhardtii L23 mutant proteins by PNGase F and labeled with 2-aminobenzamine (2-AB) after 72h alpha-mannosidase treatment.

In conclusion, N-glycan structure analyses of glycoproteins from L23 corroborate the results obtained in Chapter 4 indicating that this mutant is defective in CrManI.
On the other hand, the N-glycan structures obtained for glycoproteins from cc503 and cc1036 strains have also confirmed our previous genetic and biochemical data, with the only exception of the presence of fucosyl residues, which seem to be under-represented in the N-glycan structures analyzed so far, when compared to the results expected from fucosyltransferases gene expression as well as from α(1,3)- and α(1,6)-fucosyl residues detected by immuno- and affinoblotting. Nevertheless, more analysis are under progress to solve these discrepancies.

V. DISCUSSION

V. Discussion

Clear evidence of the existence of a route of protein transport to the chloroplast through the endomembrane system in *Arabidopsis thaliana* has been presented by Villarejo *et al.* in 2005 (Villarejo *et al*., 2005; Radhamony and Theg, 2006). Villarejo *et al.* showed that a carbonic anhydrase of *Arabidopsis*, CAH1, is located exclusively in the chloroplast stroma, despite the presence of a signal peptide for the ER. CAH1 is not imported directly into the chloroplast through the Toc/Tic system but it´s imported simultaneously with its translation into the ER and, once inside, it is processed to its mature form which includes the addition of N-glycans on up to five asparagine residues. From the ER, this protein is sorted to the Golgi apparatus, where the N-glycans are modified to obtain the typical plant complex-type N-glycan, containing β(1,2)-xylose and α(1,3)-fucose residues (Villarejo *et al.,* 2005). The protein is finally transported to the chloroplast in its folded and glycosylated mature form (Villarejo *et al*., 2005). This is the first study showing two milestones that will change our view of intracellular trafficking of proteins. First, Villarejo *et al.* (2005) demonstrated the existence of a direct connection between the endomembrane system and organelles as the chloroplast. Second, they showed the existence of N-glycosylated proteins in organelles of endosymbiotic origin, since the general dogma postulated that these proteins were not present in the chloroplast of plants. Since then, other research groups have found other chloroplast glycoproteins that appeared to follow a similar path to that described for *Arabidopsis* CAH1 in other species such as rice. Specifically, the α-amylase I-1 (Asatsuma *et al*., 2005) and nucleotide pyrophosphatase / phosphodiesterase (NPP1) (Nanjo *et al*., 2006) are glycoproteins that are localized in the chloroplast stroma of this species.

All these data indicate that the plastid proteome contains, besides the proteins synthesized in the chloroplast and those transported through the Toc/Tic complex, glycoproteins distributed through the endomembrane system (ER and Golgi) (Villarejo *et al.,* 2005; Asatsuma *et al*., 2005; Nanjo *et al.,* 2006).

From the evolutionary point of view, when the first genes were transferred from the genome of the cyanobacterial endosymbiont to the nuclear genome, there was probably no protein-sorting system for the ancestral chloroplast. The encoded proteins may have been secreted from the eukaryotic host and subsequently taken up by the endosymbiont.. This ancestral pathway seems to be maintained in the present chloroplast for some chloroplastic proteins. From that, one could postulate that this route would exist in all organisms of Viridiplantae group, including algae, mosses and land plants, among others.

The results presented in this work show that the chloroplast of *Chlamydomonas reinhardtii* contains glycoproteins as it occurs in *Arabidopsis*. Chloroplast glycoproteins found in *C. reinhardtii* contain α(1,3)-fucose and β(1,2)-xylose residues (Figure 1.1), which indicate that these proteins possess complex-type N-glycans . Fucose residues are

added, at least in plants and insects, by the action of α(1,3) fucosyltransferases localized in the Golgi apparatus and β(1,2)-xylose residues are added in plants by the action of an β(1,2)- xylosyltransferase also localized in the Golgi (Lerouge *et al*., 1998; Villarejo *et al.,* 2005; Bardor *et al.,* 2009). The latter would imply that, as in plants, glycoproteins in *Chlamydomonas* are transported to the chloroplast through the endomembrane system (ER and Golgi).

Our data obtained with several inhibitors of protein trafficking through the endomembrane system indicate that, indeed, glycoproteins of *C. reinhardtii* are transported through the ER and Golgi (Figure 1.2). Tunicamycin, which inhibits glycosylation of proteins in the ER (Misoon y col., 2004), blocks the presence of glycoproteins in the chloroplast which indicates that chloroplast glycoproteins are synthesized in the rough ER and enter in the lumen of this compartment where these glycoproteins are N-glycosylated. Brefeldin A (BFA) is a fungal inhibitor that blocks the action of Arf1, a small GTPase involved in retrograde transport from the Golgi to the ER and a subsequent total blockage of vesicular trafficking between the ER and Golgi (Ritzenthaler *et al.,* 2002). Chloroplast glycoproteins of *C. reinhardtii* seem to follow a BFA sensitive transport pathway which would imply that, once incorporated into the ER, are transported to the Golgi apparatus in vesicles. The *Arabidopsis* glycoprotein CAH1 follows an identical route (Villarejo *et al.,* 2005). These results, along with the presence of α(1,3)-fucose and β(1,2)-xylose residues in their N-glycans, confirm that the transport of such proteins occurs through the Golgi.

Although the results of Villarejo *et al*. (2005) strongly indicate that vesicular transport through the secretory system provides the mechanism by which CAH1 is transported into the plastid, it remains to be determined how precisely this is accomplished (Radhamony and Theg, 2006). Monensin is a monovalent ionophore which inhibits vesicular transport, disrupting secretory and endocytic activities of cells in culture (Ledger and Tanzer, 1984). Our results (Figure 1.2) indicate that, in the presence of monensin, glycoproteins mostly appear in chloroplast fraction. However it has also been described that monensin can cause swelling of mature cisternae of plant Golgi apparatus (Boss et al., 1984). In fact, the latter seems to be the case in our experimets using monensin to find out if glycoprotein transport to the chloroplast was mediated by vesicles in *Chlamydomonas*. Immunoblots against BiP chaperone, a protein involved in ER protein quality control, would indicate that in the cells treated with Monensin the microsomal fraction would fractionate as a contamination of chloroplast fractions, as indicated by the presence of BiP chaperone. The latter would be indicating that Monensin effect on the endomembrane system causes a dramatic effect which induces the collapse of endomembrane system and the observed changes in the pattern of fractionation of this compartment. Therefore, more work has to be made to determine how glycoproteins are transported from Golgi to the chloroplast.

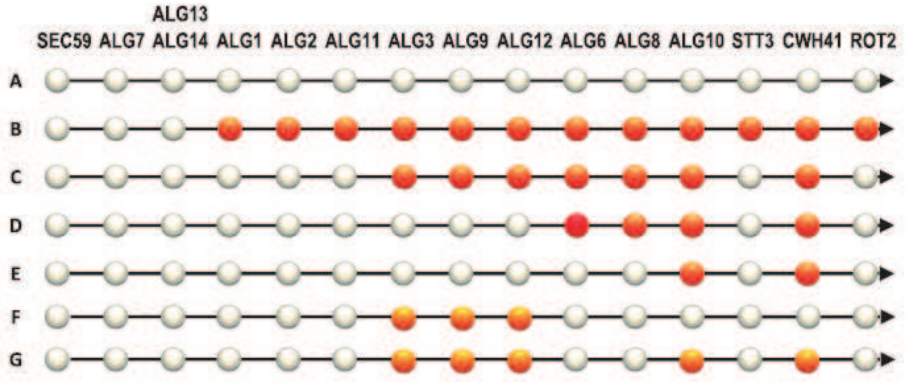
The presence in *C. reinhardtii* of an N-glycosylation pattern in the endogenous glycoproteins potentially similar to the observed in plants encouraged us to characterize the biosynthetic pathway of complex–type N-glycan in *Chlamydomonas*, as well as to analyze their structure and composition. N-glycosylation of proteins is one of the most important post-translational modifications in the maturation process of proteins in eukaryotes. This process is largely characterized in plants, mammals and insects (Lerouge *et al.,* 1998; Wilson, 2002; Bardor *et al.,* 2009). However, genetic or biochemical data on this route in green algae are almost absent and only some studies on diatoms as *Phaeodactylum tricornutum* (Baïet *et al*., 2011) or in the red algae *Porphyridium sp* (Levy-Ontman *et al*., 2011) have been recently published.

In plants, the biosynthetic pathway that transforms the high mannose type N-glycan synthesized in the ER in complex-type N-glycan has been characterized in depth in recent years (Strasser *et al.,* 2006, Strasser *et al*., 2007; Bardor *et al.,* 2009). The genes encoding the enzymes of this pathway have been cloned and a functional characterization of these enzymes has been carried out. The formation of complex-type N-glycans in plants is the result of the sequential and orderly action of several enzymes localized in Golgi apparatus, starting with the action of α-Man I and followed by the sequential activities of GnT I, α-Man II, GnT II, β(1,2)-xylosiltransferase and α(1,3)-fucosyltransferase (Lerouge *et al*., 1998; Strasser *et al*., 2006; Bardor *et al*., 2009). The combination of the last two enzymes occurs specifically in plants and the result is the typical plant complex-type N-glycan characteristic of these organisms.

The N-glycans of insects, although have α(1,3)-fucose residues, lack β(1,2)-xylose residues. Unlike plants, insects present an α(1,6)- fucosyltransferase that adds an α(1,6)-fucose residue to the proximal N-acetylglucosamine in the N-glycan core (Wilson, 2002; Paschinger *et al*. , 2005). This enzyme is also present in mammals, so N-glycan of mammals contain α(1,6)-fucose residues but not α(1,3)-fucose residues (Staudacher *et al.*, 1999).

In addition to the different composition of N-glycan observed in insects, mammals and plants, the other distinguishing feature is their biological function. Mutations altering this biosynthetic pathway in insects and mammals are lethal (Freeze, 2002). In this regard, there have been described big changes in the glycosylation pattern and branching of N-glycans attached to glycoproteins in various mammalian cancer cell lines allowing to speculate on their importance in processes such as metastasis and malignant tumors (Staudacher *et al.,* 1999). However, plants mutated in genes affecting the biosynthesis of complex-type N-glycans, as *cgl1* or *stt3a* mutants or of *Arabidopsis thaliana,* do not show an evident phenotype under non stressed conditions (von Schaewen *et al.,* 1993, Strasser *et al.,* 2004), although differences in cell-wall formation, growth inhibition, aberrant root-tip morphology, and callose accumulation, become significant under salt stress (Kang *et al*., 2008).

In silico analyses of *C. reinhartdii* genome allowed the identification of most of the genes encoding proteins involved in ER pathway of N-glycan biosynthesis, such as the Asparagine-linked-Glycosylation enzymes (ALG), the orthologs of the oligosaccharyltransferase (OST) complex subunits and enzymes involved in the maturation in the endoplasmic reticulum as well as in the protein quality control. This part of the biosynthetic pathway is highly conserved in animals, plants, fungi and protists (Kukuruzinska and Lennon, 1998) and our data confirmed this fact. However, as was mentioned in the Chapter 2 of Results (see Table 2.1) and has been previously reported by Gomord *et al.* (2010) we failed to find the Asparagine-linked-Glycosylation genes ALG3, ALG9, ALG12 and ALG10. The latter would be indicating, according to Gomord *et al.* (2010), that *Chlamydomonas* N-glycans would lack the branched antenna, which is synthesized by ALG3, ALG9 and ALG12, whereas the absence of ALG10 would determine the lack of the terminal glucose in their final ER N-glycan precursor, as it can be seen in the scheme (Figure D1) obtained from Gomord *et al.* (2010).



	A Dol-P-P	B Dol-P-P	C Dol-P-P	D Dol-P-P	E Dol-P-P	F Dol-P-P	G Dol-P-P
Plant	*A. thaliana* *A. lyrata* *O. sativa* *R. communis* *S. moellenorffii*	*NI*	*NI*	*O. tauri* *O. lucimarinus*	*M. pusilla* *P. patens?* *P. trichocarpa?*	*V. carteri*	*C. reinhartdii*
Protozoa	*N. gruber* *D. purpurumi*	*G. lamblia*	*E. histolytica* *T. vaginalis*	*T. brucei* *T. cruzi*			
Fungus	*A. brassicola* *A. niger* *N. discreta*			*C . neoformans*			
Chromist	*P. ramorum*				*P. tricornutum*		*A. anophage -fferens*

Figure D1: "Structural diversity of dolichol-linked oligosaccharides and their biosynthesis in eukaryotes. **(A)** Reference assembly pathway and oligosaccharide donor structure found in most eukaryotes. (B–G) Truncated dolichol-linked oligosaccharides are made in some eukaryotes, after secondary loss of ALG genes (in red in the upper panel)." Taken from Gomord et al.(2010).

Despite of the latter, it has to be noticed that our analyses of the complex-type N-glycan structures from the different strains of *Chlamydomonas* used in this study, clearly indicate the presence of Man5GlcNac2 structures, compatible with a triantennary structure of the precursor after maturation in the Golgi. In fact, the N-glycans from L23 mutant, which does not present α-Mannosidase I activity, exhibit N-glycans going from Man-9 to Man-5, reinforcing the existence of this triantennary high-mannose type structure. We can speculate that these enzymes, although present in *Chlamydomonas*, could not be found by different reasons, being one possibility the presence of wrong annotations in some genomic regions of the published version of *Chlamydomonas*. Another possibility could be that the genes coding for these enzymes would not share enough homology to be identified by sequence similarities to their respective ALG yeast homologs used as reference. In fact, a functional homolog of the ALG3 mannosyltransferase, which is responsible for the first mannosylation step of the lipid-linked *N*-glycan in the ER lumen, was recently identified and characterized in *Arabidopsis* (Henquet *et al.*, 2008). Interestingly, and by contrast with plant treatment with tunicamycin, a knockout of ALG3 activity was not lethal in *Arabidopsis* mutants. The recent identification of several homologs of yeast ALG glycosyltransferases in the genome of *Arabidopsis* (Gomord *et al.*, 2010) now offers opportunities for detailed studies on the physiological importance of the different glycosyltransferases involved in the dolichol pathway in plants as well as in other organisms.

The *in silico* analyses of the genes coding for Golgi localized enzymes involved in the maturation of the *Chlamydomonas* N-glycans, revealed the presence of one Golgi α-Mannosidase I (CrManI), α-Mannosidase II (CrManII), β(1,2)-xylosiltransferase (CrXylT), α(1,3)-fucosyltransferase (CrFT1) and a α(1,6)-fucosyltransferase (CrFT8). Surprisingly, the genome of *Chlamydomonas reinhardtii* don´t seem to present orthologs of the other basic enzymes involved in the biosynthesis of complex-type N-glycans in plants, mammals and insects. Focusing on the comparison with the N-glycan of plants, the most relevant result is the absence of orthologous sequences of GnTI and GnTII. These data suggest that N-glycan of this organism is different and much simpler than the N-glycan described in other organisms.

Characterization of α-Mannosidase I (CrManI) of *C.reinhardtii* showed that CrManI presents homology with other α-Mannosidases I from the GH47 family described in CAZy database (http://www.cazy.org/) enting 37% of sequence identity with *Arabidospis thaliana*; 35% with *Droshopila melanogaster* and 51% of identity with *Homo sapiens* (Figure 2.1B). The catalytic domain of CrManI contains the three highly conserved sequence motifs characteristic of Class I α-Mannosidases and the Threonine (T) residue of the motif III (Tempel *et al*., 2004; Kajiura *et al.,* 2010) as well as the acidic residues and two of the cysteine residues (Cys-427 and Cys-462) essential for α(1,2) mannosidase activity (Lipari and Herscovics, 1996, 1999) (Figure 2.1B). In addition, CrManI exhibit the expected transmembrane domain in its N-terminus characteristic of the type II membrane protein topology inherent to this enzyme family.

This enzyme catalyze the first step in the modification of the high mannose-type N-glycan synthesized in the ER, which consists in removing a variable number of α(1,2) linked mannoses from the N-glycan antennae, yielding simpler molecules. Our data obtained from the L23 mutant of *Chlamydomonas*, which is defective in *crmanI* (see Figures 4.7 and 4.6), confirm that this gene is coding for an α(1,2) mannosidase I, as has been demonstrated by mass sprectrometry N-glycan analyses (see Figures 5.6 and 5.7) indicating the presence of additional mannose residues in the N-glycans from this strain when compared to those of the wt cc503 or its parental strain cc1036.

Regarding to the putative α-Mannosidase II (CrManII) identified in this study (see Figure 2.2B), it is a large protein (1359 aminoacid) containing the three Pfam domains described in CAZy database for this GH38 family of glycosyl hydrolases as well as the conserved residues involved in Zn^{2+} binding in the catalytic site, as has been described for Mannosidase II of *D.melanogaster* (van den Elsen *et al*., 2001). When the sequence homology with other Golgi localized α-mannosidases is analyzed CrManII presents 34% of sequence identity with *Arabidopsis thaliana*; 35% with *Droshopila melanogaster* and 50% of identity with *Homo sapiens* (Figure 2.2B), which are on the same range of sequence identity found for the other Golgi enzymes identified. However, it has to be said that this protein does not present a clearly specified N-terminal hydrophobic domain, as should be expected for a type II membrane protein localized in the Golgi. The latter could indicate that may be this protein could be a α-mannosidase II belonging to the soluble cytosolic machinery for N-glycan recycling, as has been suggested by Dr. E. Rivet and Dr. M. Bardor (personal communication) after a preliminary analysis of sequence similarities comparing CrManII to ManII enzymes from different organisms and cell localizations (lysosomes, Golgi and citosol), in the frame of their committed objectives in the consortium for the ALGALGLYCO project.

CrXylT, the putative β(1,2)-xylosiltransferase identified in *C.reinhardtii*, presents a high homology with β(1,2)-xylosyltransferases from other organisms (see Figure 2.3B), all belonging to the GT61 family of glycosyltransferases described in CAZy database (http://www.cazy.org/) confirm the type II membrane domain in this protein, there is a predicted transmembrane domain from residue 3 to 24 in the N-terminus of CrXylT, which overlaps with the CT conserved region previously described for other XylT (Bencur *et al.,* 2005). However, neither the proline-rich region nor the KPWP sequence, suggested as important for XylT activity in *Arabidopsis thaliana* (Pagny *et al.,* 2003; Léonard *et al.*, 2004) are well conserved in *Chlamydomonas*. Nevertheles, our data clearly confirm this activity in the three strains of *Chlamydomonas*, since we have confirmed the presence of β(1,2)-xylose residues by different techniques, as immunobloting analyses (see Figure 4.4) and from the MALDI-TOF MS (see Figures 5.1 to 5.4 and Figures 5.6 and 5.7) and MALDI-TOF MS-MS (see Figure 5.5) N-glycan analyses from the three strains. It has to be mentioned that immunoblotting analyses against β(1,2)-xylose residues in the cell wall less cc503 strain (see Figure 4.4) indicated that this strain seem to present a lower content of this residues when compared to the other two strains analyzed, probably reflecting the lack of the N-

glycoproteins which are the main components of *Chlamydomonas* cell wall (Catt *et al*., 1978).

On the other hand, data from analyses MALDI-TOF MS-MS after permethylation of complex-type N-glycans in the three strains, which have been performed by Dr. M. Bardor (Figure 5.5 and personal communication) reveal the presence of a Xylosyl terminal residue, different to the β(1,2)-linked to the core mannose. The specific linkage of these xylosyl residues is still unknown and more analyses have to be made to solve this question as well as the possibility to find a new xylosyltransferase encoded by *Chlamydomonas* genome.

CrFT1, the α(1,3)-fucosyltransferase identified in *C. reinhardtii* exhibits 31% of identity with *Arabidospis thaliana* FT12; 34% of identity with *Arabidopsis thaliana* FT11 and 27% with *Droshopila melanogaster* (see Figure 2.4B), all of them α(1,3)-fucosyltransferases from the GT10 family of glycosyltransferases described in CAZy database (http:// www.cazy.org). The sequence identity among these α(1,3)-fucosyltransferases is low but the catalic domain II and a motif SNC(G/A)A present a very high homology described for this group, the one distinguishing feature is the absence of the motif I (SSDV), this motif neither present in the α(1,3)-fucosyltransferase of animals, diatoms and insect (see Figure 2.4B). As was previously mentioned, CrFT1 does not completely fulfill the requirements of hydrophobicity to safely predict a transmembrane domain in its N-terminus. Nevertheless this is also the case for the already well characterized FT1 from *D. melanogaster.*

The number of different α(1,3)-fucosyltransferases that are present in a organism varies greatly among different kingdoms. *Arabidopsis thaliana* possess two genes coding for α(1,3)-fucosyltransferases, *AtFT12* and *AtFT11* (Bakker *et al.,* 2001) the two enzymes exhibiting around 80% homology. Results obtained in our laboratory indicate that both α(1,3)-fucosyltransferases in *Arabidopsis thaliana* are functional and involved in the fucosylation of proteins according to their final destination (Villarejo *et al.,* unpublished data). In the nematode *Caenorhabditis elegans* have been discovered up to 18 different genes encoding putative α(1,3)-fucosyltransferases of which only one has been cloned (Oriol *et al.,* 1999). *Chlamydomonas reinhardtii* has a unique α(1,3)-fucosyltransferase. Our data show that gene expression of c*rft1* is tightly regulated by growth conditions. Changes in CO_2 concentration in the medium, cause drastic changes in the expression of this gene, so that its expression is suppressed completely in cells growing for 6 h in low CO_2. This suppression, however, is transient and may be due to stress experienced by the cells at the time of transfer to nutrient limited conditions. In *Arabidopsis, AtFT11* and *AtFT12* expression appears to be constitutive and unaffected by environmental factors or by the development stage of the plant. Thus, the differential expression of CrFT1 depending on environmental conditions seems to be a specific feature of *C. reinhardtii crft1* which is not present in other photosynthetic organisms. In contrast, fucosylation levels and therefore the expression of α(1,3)-fucosyltransferases

in vertebrates and invertebrates are affected by physiological and developmental state (Staudacher *et al*., 1999).

The formation of complex-type N-glycans in most eukaryotes, including plants, animals and insects, requires the enzyme GnTI (von Schaewen *et al*., 1993, Strasser *et al.,* 2005). The action of this enzyme is a prerequisite for the action of other glycosyltransferases that operate later in the Golgi apparatus, including α(1,3)-fucosyltransferases and β(1,2)-xylosyltransferases of plants and insects as well as α(1,6)-fucosyltransferases of insects and mammals. In fact, glycoproteins of the *cgl1* mutant of *Arabidopsis thaliana,* defective in GnTI, exclusively present high-mannose type N-glycans, (von Schaewen *et al*., 1993) and it can be observed in Figure 4.4A and B.

The presence in *Chlamydomonas reinhardtii* of fucose residues attached to the N-glycan via a α(1,3)- linkage, together with the absence of an orthologous sequence of GnT I in its genome, suggested the existence in this organism of a fucosylation pathway independent of GnT I. GnT I independent fucosylating activities have been previously described in the nematode *Caenorhabditis elegans* (Paschinger *et al.,* 2004) and in *Drosophila melanogaster* (Sarkar *et al.,* 2006). Nevertheles, these differences in substrate specificity of α(1,3)-fucosyltransferases among plant and mammalian enzymes in one side and animal invertebrates in the other, are not reflected in large differences in sequence or presence of specific domains (see Figure 2.4B).

Although in nematodes and insects the α(1,3)-fucose is added to the proximal N-acetylglucosamine of N-glycan core, our results indicate that this α(1,3)-fucose residue of *Chlamydomonas reinhardtii* is added in a different position. The pattern of digestion of N-glycans anchored to glycoproteins with commercial glycosidases, as EndoH or PNGase F, is used routinely in the field of glycobiology as a tool to elucidate the structure and arrangement of sugar residues in the glycans of many organisms. It has long been known that the presence of a α(1,3)- fucose residue attached to proximal N-acetylglucosamine in the N-glycan core is an impediment for the action of glycosidase PNGase F (Tretter *et al.,* 1991) and N-glycans from plants and insects, which have this type of residue, are resistant to digestion with this glycosidase. On the other hand, complex-type N-glycans, where mannose residues of the antennae are substituted by residues of N-acetylglucosamine, galactose or other sugars, are resistant to the action of glycosidase Endo H.

The N-glycans attached to glycoproteins in *C. reinhardtii* are sensitive to PNGase F (see Figure 3.7). This feature clearly confirms our hypothesis that α(1,3)-fucose residue is not linked to the proximal N-acetylglucosamine of the chitobiose core of the N-glycan. Moreover, resistance to Endo H of the N-glycans from *C. reinhardtii* indicates that the mannose residues of the antennae are substituted with some type of sugar. Another explanation is that the cleavage site of this glycosidase, the O-glycosidic bond located between the two N-acetilglucosamines in the N-glycan core, was blocked

with an additional residue. A residue of this type is not described in most organisms where the N-glycan structure has been studied, including plants, animals and insects. Phospholipase A2 from bee venom has N-glycan with two fucose residues in the proximal N-acetylglucosamine on the chitobiose core (Kubelka *et al.,* 1993). However, it is in nematodes where we can found more odd glycosylation patterns. The nematode *Haemonchus contortus* has a unique pattern of fucosylation (Figure D2) (Haslam *et al.,* 1996). In this organism there are three fucose residues attached to the N-glycan core. Two of them, are located in α(1,3)-link and α(1,6)-link to the proximal N-acetylglucosamine . The third position is located in α(1,3)-link to the distal N-acetylglucosamine (Haslam *et al.,* 1996). This high degree of substitution in the N-glycan core has not been observed in any other eukaryotic glycoprotein. N-glycans of such organism are paucimannosidic type, lacking N-acetylglucosamine in the antennae of the N-glycan but containing 3 mannose residues attached to the hyper-fucosylated chitobiose, therefore allowing the affinodetection by the lectin Concanavalin A (ConA) (Haslam *et al.* 1996).

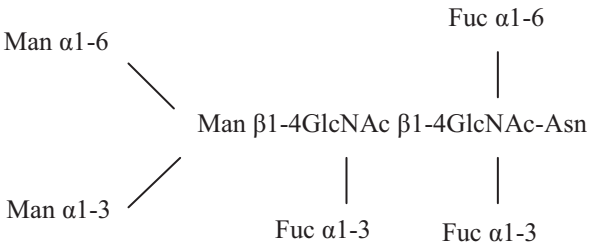


Figure D2: "Pattern of fucosylation in nematode *Haemonchus contortus*" Taken from Haslam *et al.,* (1996)

The absence of N-acetylglucosamine residues in these glycans allowed us to deduce that the addition of fucose residues takes place through a pathway independent of GnT I, similar to that described for other nematodes as *C. elegans* (Paschinger *et al.,* 2004). The N-glycan of the nematode *H. contortus* has similar characteristics to those described for *C. reinhardtii* in this work. Therefore, one might speculate that the N-glycan of *C. reinhardtii* would be similar to this nematode except for the absence of α(1,3)-fucose attached to the proximal N-acetylglucosamine. The presence of α(1,3)-fucose attached to the distal N-acetylglucosamine would block the action of Endo H glycosidase and the presence of terminal mannoses in the antennae would allow binding to ConA, as observed in our study.

As we mentioned above, most of the α(1,3)-fucosyltransferases of plants, animals and insects have the same acceptor specificity on which the substrate GDP-fucose is added. This acceptor usually includes a free terminal N-acetylglucosamine (Oriol *et al*., 1999, Bakker *et al*., 2001, Wilson *et al.,* 2001). The differential features of CrFT1 suggested that the acceptor specificity of this enzyme seems to be GnT I independent and different to most of the α(1,3)-fucosyltransferases from other organisms. The results obtained in protoplast transfection experiments in *Arabidopsis*

confirmed that (see Figure 3.8) CrFT1 can partially restaure the addition of α(1,3)-fucose residues to the N-glycans present in the double mutant of *Arabidopsis thaliana* ft11/ft12. The N-glycans of this mutant lack α(1,3)-fucose residues but have β(1,2)-xylose and N-acetylglucosamine in the terminal end of the N-glycan (Villarejo *et al.*, unpublished data). However, CrFT1 is more efficient adding α(1,3)-fucose residues to the N-glycans of cgl1 mutant of *Arabidopsis*, which lack the terminal N-acetylglucosamine. The α(1,3)-fucosyltransferases of plants and mammals are unable to add the substrate to acceptor molecules lacking terminal N-acetylglucosamine (Bakker *et al*., 2001, Wilson *et al.,* 2001) which is in contradiction to what we observed with CrFT1 of *Chlamydomona*s. Only the α(1,3)-fucosyltransferases of some invertebrates are able to use this type of acceptor molecules (Paschinger *et al.,* 2004). Our results suggest that the enzyme CrFT1 of *Chlamydomonas* is more versatile than the present in plants and mammals. Silencing of *crft1* gene by artificial microRNAs in *Chlamydomonas* further confirm (see Figures 3.10 and 3.11) that CrFT1 is responsible for the addition of α(1,3)- fucose residues since the silencing of this gene is reflected in a decrease in the level of fucosylation of the endogenous algal N-glycoproteins. The N-glycan analysis from glycoproteins of the FT1-3 7 H silenced line, which is under progress in the laboratory of Dr. M. Bardor, should confirm our genetic and biochemical data. Nevertheless, it has to be mentioned that the data obtained so far regarding to the presence of fucose residues in MS N-glycan analyses reveal a lower fucose content in *Chlamydomonas* N–glycans than it can be expected from immunoblot analyses against specific α(1,3)fucose antibodies.

Another aspect that remains to be elucidated is the presence of additional residues in the N-glycan attached to endogenous glycoproteins of *C. reinhardtii.* Since *Chlamydomonas* N-glycans were resistant to Endo H digestion (see Figure 3.7), the presence of a residue blocking the action of this glycosidase was considered. If the N-glycan of *C. reinhardtii* were similar to the nematode *H. contortus*, one would expect to find a residue of α(1,6)-fucose in the proximal N-acetylglucosamine of the N-glycan core. The presence of α(1,6)-fucose residues linked to the proximal N-acetylglucosamine in the N-glycan core is a typical characteristic of mammals and insects (Staudacher *et al*., 1999; Paschinger *et al*., 2005) and has never been found in plants (Lerouge *et al*., 1998; Bardor *et al*., 2009).

In silico analysis of *Chlamydomonas* genome revealed the presence of one gene, *crft8*, encoding for a putative α(1,6)-fucosyltransferase. This gene presents 8 exons and 7 introns (Figure 3.13A). α(1,6)-fucosyltransferases belong to the GT23 family of glycosyltransferases in CAZy classification. These family of enzymes are typical type II membrane proteins, localized in the Golgi apparatus. Nevertheless, the putative CrFT8 protein doesn´t seem to exhibit the appropriate transmembrane domain according to the SOSUI server prediction as well as occurs in FT8 protein from *Ciona intestinalis* (Figure 3.13B). The putative CrFT8 presents the Motif I, conserved in insects and other organisms, which is essential for activity, as well as the conserved motifs II and III (Figure 3.13B).

We found that *crft8* gene is expressed (see Figure 3.14) and we detect α(1,6)-fucose residues (see Figure 3.16) in the endogenous algal N-glycoproteins by affinoblotting using the lectin AAL (biotinylated *Aleuria aurantia* Lectin). Therefore, *crft8* seems to be a functional gene in *Chlamydomonas*. However much work needs to be made in order to confirm the activity of this protein, since we have failed to find α(1,6)-fucose residues in any of the Mass Spectrometry N-glycan analyses performed so far. Regarding to that, and based on the common features shared by α(1,6)-, α(1,2)- and O-fucosyltransferases (Oriol *et al.,* 1999; Takahashi *et al.,* 2000; Chazalet *et al*., 2001; Martinez-Duncker *et al*., 2003) we have to consider the possibility of this enzyme as adding fucose in structures different from N-linked glycans.

This composition of the N-glycan of glycoproteins *Chlamydomonas*, with α(1,3)-fucose, β(1,2)-xylose and α(1,6)-fucose residues, suggests that glycosylation in this alga is different from the present in higher plants (Wilson, 2002).

The characterization of L23 mutant made us to consider again the possibility of the presence of additional residues in the N-glycan attached to endogenous glycoproteins in *C. reinhardtii* Our results clearly demonstrate that this mutant is defective in the enzyme α-mannosidase I, resulting in the accumulation of high mannose type N-glycans. The lack of CrManI in the mutant also justifies the high mannose content which was detected in the monosaccharide composition of flagellar glycoproteins (Bloodgood, 1988). However, this defect also causes the absence in the mutant N-glycan of an epitope recognized by the FMG-1 monoclonal antibody, whose nature is unknown at the moment. Our results show that this epitope is absent in *Drosophila melanogaster* and *Arabidopsis thaliana* (see Figure 4.2) as well as in multicellular species of the genus *Volvox* and *Pandorina* (Gimmler and Bloodgood, personal communication). In addition, this epitope not only recognized a glyco- epitope linked to flagellar glycoproteins in *Chlamydomonas* N-glycans, but it recognizes N-intracellular N-glycoproteinas. Although FMG1 antibody could be recognizing the structure of the entire N-glycan, another feasible option is to recognize a residue still to be identified, which would be absent or hidden as a consequence of the non-function of mannosidase I.

Although N-linked glycosylation and N-linked glycan structure have been well studied in mammals and other eukaryotes, very little attention has been paid to studying N-linked glycosylation in algae. In a recent study, Baïet *et al*. (2011) present an analysis of N-glycan biosynthesis and structure from the diatom *Phaeodactylum tricornutum*. Results of this analysis have demonstrated that glycoproteins from this alga carry mostly high mannose type N-glycans ranging from Man-5 to Man-9. In addition, minor glycans Man-3 and Man-4 carrying some α(1,3)- linked fucose have been identified. β(1,2)-xylose seem to be absent in *P.tricornutum* proteins. In addition, these authors have performed a through genetic and functional characterization of GnT I, demonstrating the *in vivo* activity of the putative enzyme by expressing the full-length

protein in Chinese hamster ovary (CHO) Lec1 mutant (lacking its endogenous GnT I activity). However, despite of the presence of a functional GnT I, as well as an α(1,3)-fucosyltransferase, no glycans carrying terminal GlcNAc residues has been detected on *P. tricornutum* proteins and only a few Man3FucGlcNAc2 carrying a fucose α(1,3)-linked. Baïet *et al.* (2011) propose that GlcNAc and α(1,3)fucose are residues susceptible of post-maturation processes in the Golgi and thereafter, as has been demonstrated in other organisms as insects (Altmann *et al.*, 1995), *C. elegans* (Zhang *et al.*, 2003) or plants (Vitale and Chrispeels, 1984).

The mass spectrometry analyses of N-glycans from *Chlamydomonas reinhardtii* also present a lower amount of ions including α(1,3)-fucose, despite of the presence and expression of CrFT1, as well as the clear detection of this sugar residue by western blot. And the same could be possible for α(1,6)-fucose residues. Therefore a search for genes coding for such fucosidase activities has to be made to answer this question.

The N-glycan analyses performed in this work show differences between *Chlamydomonas* structure and lands plants, and confirm some of the biochemical and genetic data obtained in this work. The presence of oligomanosidic structures similar to those found in plants and other organisms confirms our identification of an ortholog of the enzyme α-Mannosidase I. This enzyme would be responsible for the stepwise removal of 1 to 4 mannose residues from the high-mannose N-glycan coming from ER yielding different oligomannoside intermediates. Furthermore, these structural data also confirm the presence of β(1,2)-xylose linked to the core mannose and α(1,3)-fucose residues in the N-glycans of *Chlamydomonas,* we had been shown by genetic and immunological techniques.

Preliminary gas chromatography analysis had also shown the presence of 6-O-methylmannose and xylose in the monosaccharide composition obtained from *Chlamydomonas* N-glycans. Experiments of MS-MS after permethlylation allowed us to position one xilose residue on the first mannose from the core of the N-glycans with link β(1,2) and a second one in the terminal position of the oligomannosides with link yet not determined (Figure 5.5). Levy-Ontman *et al*. (2011) studied the structure of the N-linked glycans in the 66-KDa glycoprotein of the red microalga *Porphyridium* sp. This study supply the first report of N-glycans with a terminal xylose attached to the 6-mannose branch of the 6-antenna and another one attached to the 3-oxygen of the distal GlcNAc in the core. Another novel finding was that all glycans contain three O-methylmannose residues in positions that have never been reported before. While it is known that some lower organisms (nematodes, yeasts, snails, algae and planarians) are able to methylate terminal monosaccharides in N-glycans, these study on P*orphyridium* sp. described for the first time a process of internal methylated mannose residues. We obtained similar results for *C.reinhardtii* (Figure D3).

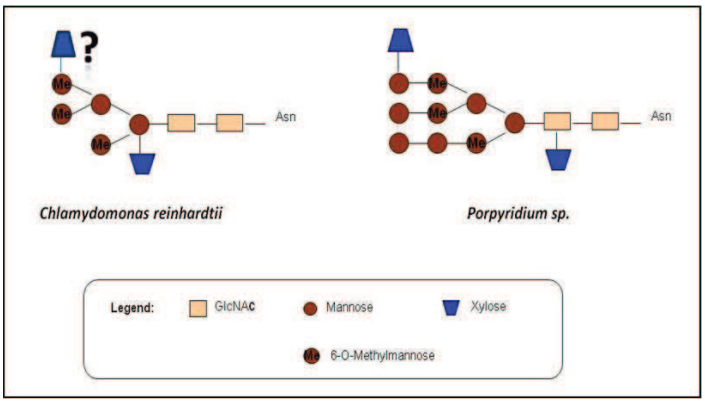


Figure D3: Suggested structure of the N-glycan in *C.reinhardtii* and the 66-kDa cell wall glycoprotrein from the red microalga *Porphyridium sp*.

On the other hand, we demonstrate that L23 is bearing higher number of mannose residues in their N-glycan oligomannosides when compared to those of the wild-type and cc1036 (see Figure 5.6). The N-glycan structures observed for the L23 mutant are going from Man5 to Man9 (and even some Man-10 and Man 11) which is in agreement with the mutation in the mannosidase I gene. The L23 oligomannosides are also bearing one or two xylosyl residues as observed for the cc1036 and the cc503 strain, but are not methylated.

Recently, Mamedov and Yusibov (2011) have described the presence of sialylated glycoproteins in the unicellular green algae *C.reinhardtii*. Their data suggest that *C.reinhardtii* has mammalian-like N-linked glycans, with (1,4)-Gal associated with a sialylated complex glycan structure and a plant-like core 1,3 fucosylation, however they did not found any putative gene homolog for mammalian sialytransferases in the *C.reinhardtii* genome database. The presence of any xylose-containing oligosaccharides in *C.reinhardtii* has not been confirmed in this study either. We have demonstrated the presence of xylose residues using both mass spectrometry and biochemical analyses as well as the presence of the putative gene *crxylt*, coding for a CrXylT and 6-O-methylmannose residues in the N-glycan structure of *Chlamydomonas*.

Given that green algae such as *C. reinhardtii* are on the basis of the evolution of the lineage that led to the existing plants, our results imply that during the evolution of this lineage would have been great changes in a process so fundamental for the structure and function of proteins as it is N-glycosylation. The organisms of the chlorophyta the *Chlorophyta* group (green algae, including *Chlamydomonas*) diverged from the *Streptofitas* (land plants and their closest relatives) billion years ago. These two lineages are part of the green lineage (*Viridiplantae*), which had previously diverged from the *Opistokontes* (animals, fungi). Sequencing the genome of *Chlamydomonas reinhardtii* revealed that many genes of this organism can be traced to the common ancestor of plants and animals (Merchant *et al*., 2007). The presence of an N-glycosylation process similar between green algae and some belonging to the *Opistokontes* reveal that some of these features have been lost in land plants.

The results obtained so far allow us to unravel the N-glycans biosynthesis in the green alga *C.reinhardtii.* Interestingly, this pathway is quite different to the well-known of higher plants, even if *C.reinhardtii* is closely related to land plants in terms of evolution. Now, it would be relevant to understand how N-glycosylation process has evolved in eukaryotes. The discovery in *C. reinhardtii* of a new route to transport glycoproteins to the chloroplast through the endomembrane system, similar to the one discovered in *Arabidopsis thaliana* (Villarejo *et al*., 2005) is of great relevance, since the chloroplast of *C. reinhardtii* can be used as biofactory for the expression of recombinant glycoproteins in this organism. The combination in the same organism of a pathway of N-glycosylation of proteins and a route that would allow to accumulate heterologous glycoproteins in the chloroplast, make this organism a very good candidate to be used as biofactory for the production of recombinant glycoproteins. Furthermore, *Chlamydomonas* have quite a number of additional advantages over other systems currently used, as being a GRAS organism (Generally Regarded As Safe), with fast grow and easy to scale-up, low production costs, rapid generation of transgenic lines, etc… that make worthy to follow the present line of research in order to finally produce therapeutic recombinant glycoproteins in this green alga.

VI. CONCLUSION

VI.Conclusion

1. The chloroplast of *Chlamydomonas reinhardtii* contains glycoproteins as it occurs in plants. Chloroplast glycoproteins found in *C.reinhardtii* contain α(1,3)-fucose and β(1,2)-xylose residues, which indicate that these proteins possess complex-type N-glycans. Glycoproteins of *C. reinhardtii* are transported through the endomembrane system (ER and Golgi), where the N-glycans are synthesized and post-traslationally modified.

2. *In silico* anal*yses of C.reinhardtii* genome allowed the identification of most of the genes encoding proteins involved in the endoplasmic reticulum pathway of N-glycan biosynthesis, such as the Asparagine-linked-glycosylation enzymes (ALG), orthologs of the oligosaccharyltransferase (OST) complex subunits and enzymes involved in ER maturation as well as in protein quality control. We failed to find the Asparagine-linked-Glycosylation genes coding for ALG3, ALG9 and ALG12, required to add the mannose residues to synthesize the branched antenna and ALG10 which add the terminal glucose residue of the oligosaccharide precursor. However, data from N-glycan structure analysis clearly indicate that *Chlamydomonas* N-glycans do present a triantennary structure.

3. *In silico* analyses of the genes coding for Golgi localized enzymes involved in the maturation of *C.reinhardtii* N-glycans, revealed the presence of one Golgi α-Mannosidase I (CrManI), α-Mannosidase II (CrManII), β(1,2)-xylosiltransferase (CrXylT), α(1,3)-fucosyltransferase (CrFT1) and α(1,6)-fucosyltransferase (CrFT8). The genome of *Chlamydomonas reinhardtii* don´t present orthologous genes coding for GnT I and GnT IIbasic enzymes involved in the biosynthesis of complex-type N-glycans in plants, mammals and insects.

4. *Chlamydomonas reinhardtii* has a unique α(1,3)-fucosyltransferase. CrFT1 presents a partial homology with α*(1,3)-fucosylt*ransferases from other organisms. However, the catalytic domain II and a motif SNC(G/A)A are totally conserved in CrFT1. The one distinguishing feature is the absence of the motif I (SSDV), which is not present either in α(1,3)-fucosyltransferases of animals, diatoms or insects. The gene expression of c*rft1* is regulated by CO_2 concentration. The differential expression of CrFT1 depending on environmental conditions seems to be a specific characteristic of *C. reinhardtii* which is not present in other photosynthetic organisms.

5. Unlike plant, mammals and insects, catalityc activity of CrFT1 is independent of GnT I, as it occurs in some invertebrates. Moreover, the N-glycans attached to glycoproteins in *C.reinhardtii* are sensitive to PNGase F which indicate that α(1,3)-fucose residues are not linked to the proximal N-acetylglucosamine in the chitobiose core of the N-glycan. However, heterologous expression of *crft1-HA*

in mesophyll protoplasts from mutant lines of *Arabidopsis thaliana*, demonstrate that CrFT1 is able to fucosylate the same acceptor that GnT I-dependent α(1,3)-fucosyltransferases, although it is more efficient adding α(1,3)-fucose residues when the N-glycan acceptor lacks the terminal N-acetylglucosamine.

6. Silencing of c*rft1* gene by artificial microRNAs in *Chlamydomonas* further confirm that CrFT1 is responsible for the addition of α(1,3)-fucose residues since the silencing of this gene is reflected in a decrease in the level of fucosylation of the endogenous algal N-glycoproteins.

7. *In silico* analysis of *Chlamydomonas* genome revealed the presence of one putative gene, *crft8*, encoding for a putative α(1,6)-fucosyltransferase. The putative CrFT8 presents partial homology with α(1,6)-fucosyltransferases from other organisms. However, sequence conserved motif I, which is essential for activity, and motifs II and III are essentially conserved. *crft8* gene is expressed and α(1,6)-fucose residues are detected in the endogenous algal glycoproteins by affinodetection using AAL lectin. Therefore, *crft8* seems to be a functional gene in *Chlamydomonas* although more work has to be made to confirm its acceptor specificity.

8. L23 mutant of *C.reinhardtii,* the only mutant affected in the N-glycosilation pathway in *Chlamydomonas,* is defective in the enzyme α-Mannosidase I (CrManI) since L23 mutant presents a deletion in at least part of the coding sequence of *crmanI* Immuno- and affino-detection of α(1,3)-fucose , β(1,2)-xilose and α(1,6)-fucose residues is not negatively affected by the absence of CrManI enzyme in this mutant. However, this defect causes the absence, in the N-glycans from mutant cells, of a carbohydrate epitope recognized by FMG-1 monoclonal antibody, which is specific of *Chlamydomonas* and yet not determined.

9. N-glycan analyses *of C.reinhardtii* by Mass Spectrometric techniques show differences between this green alga and land plants and confirm some of the biochemical and genetic data obtained in this work. Major ions correspond to high-mannose type N-glycans, specially Man5GlcNac2, although minor ions corresponding to complex-type N-glycans containing 1 or 2 xylose residues and fucose have been found. The presence of oligomannosidic structures similar to those found in plants and other organism confirms our identification of an ortholog of the enzyme α-Mannosidase I. Experiments of MS-MS after permethylation allowed to position one xilose residue on the first mannose from the core of the N-glycans with link β(1,2) and a second one in the terminal position of the oligomannosides with link yet not determined. The most striking result is the presence of 6-O-methylmannose in the monosaccharide composition obtained from *Chlamydomonas* N-glycans.

10. L23 is bearing a higher number of mannose residues in its oligomannosides as compared to those from cc1036 or cc503 wild type strain. Indeed, the N-glycan structures observed for the L23 mutant are going from Man-5 to Man-9 and even higher, which is in agreement with the mutation in *crmanI* gene. The L23 oligomannosides are also bearing one or two xylosyl residues as observed for cc1036 or cc503 strains, but are not methylated.

VII. REFERENCES

VII. References

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